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Plant Tissue Culture: providing strategic support for the banana industry

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Queensland Government
Department of Primary Industries and Fisheries

Banana Industry Committee

HAL
Know-how for Horticulture™

BIPB Queensland

Queensland Government
Department of Primary Industries and Fisheries

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Report Purpose:
In line with HAL project guidelines, this report provides a project outline including technical summary or aims, outcomes and recommendations related to banana tissue culture use and potential in Australia. This project considers both research and industry development issues.

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Media Summary

The Australian banana industry is one of the largest horticultural fruit commodities valued at approximately M$392 for 2006-07. It has and continues to face emerging disease and market challenges that must be reduced or eliminated by developing and implementing improved farm practices. There are serious pathogens affecting commercial banana production. Subtropical Panama (*Fusarium oxysporum* f.sp. *cubense*) and Banana Bunchy Top Virus are found in areas of Northern New South Wales and South East Queensland. An even more serious pathogen, Tropical Panama, in the Northern Territory can decimate tropical banana production where, in Australia, over 80% of bananas are grown. One of the aims of this project is to provide to industry ways to eliminate entry of pests and diseases and provide means to reduce impacts of diseases where they occur. Another aim is to ensure industry has access to, and makes best use of, clean tissue culture planting material via the Quality Banana Approved Nursery scheme. Clean tissue cultured plants can be used to improve farm practice, reduce labour costs and improve product and profits to the grower. This project assists industry to meet these challenges by undertaking applied plant biotechnology research and providing support to biosecurity strategies and associated activities.

This project delivers a suite of strategic activities using plant tissue culture to provide access to specialised laboratory and quarantine facilities as well as the Australian banana germplasm collection. An AQIS registered quarantine laboratory ensures that banana varieties can be safely imported into Australia as tissue cultured plants. Approximately 400 accessions of virus indexed banana cultivars have been imported this way and are safely maintained in a tissue culture facility away from pests, diseases and severe weather events. This world recognised collection is used in research and industry development as well as in biosecurity applications.
Research has been undertaken to overcome problems and improve commercial tissue culture production in the Quality Banana Approved Nursery scheme. Grower use of tissue culture is increasing rapidly and we are undertaking research to improve production as well as to improve plant quality. Future work includes identifying beneficial bacteria that may be used to increase plant growth and improve plant defence so that plants will require less inputs for increased yield.

Technical Summary

The Australian banana industry has identified a suite of strategic activities that can only be achieved by utilising banana tissue culture. Australia has relied on plant tissue culture in this strategic way for many years and a close working relationship has resulted in world class biotechnology skills and facilities designed to support the Australian industry. By continuing to use and develop our banana tissue culture expertise, this project focuses on aspects of research and development needed to sustain the Australian banana industry in both the short term and long term. Activities undertaken in this project are assigned high priority in the current industry strategic plan.
Some of the key activities undertaken in this project will be to provide Australia with a quarantine banana importation laboratory to retain safe access to valuable new varieties. These varieties are used to support research into disease resistance, reduced environmental impacts of banana cultivation, improved farm practice and market expansion/diversification. Outcomes from this project will be to maintain and improve international and domestic banana biosecurity, maintain and provide banana varieties to encourage and support research, industry development and sustainability, undertake research to encourage wider adoption of tissue culture by identifying obstacles to production or plant quality. These activities enable industry to overcome obstacles imposed by pests and diseases, environmental and market forces.

The Australian banana industry is highly regulated with many domestic quarantine zones and restrictions to contain, reduce and restrict pest and disease. We use plant tissue culture to support our domestic quarantine. Australian now has the best disease free planting material scheme in the world. The Quality Banana Approved Nursery (QBAN) scheme is a scheme based on accredited commercial tissue culture laboratories and nurseries that use virus-indexed suckers to produce disease free plants. Staff in this project supports the ongoing development of the QBAN scheme to encourage uptake of disease free planting material for development of sustainable production practices.

Banana tissue culture facilitates research and development in the Australian banana industry's highest priorities: maintaining domestic and international biosecurity, maintaining an environmentally sustainable and viable industry and to be positioned to capture and develop markets.

While the project is based in Nambour activities are also carried out at various locations across the state. The key tissue culture research occurs in an internationally recognised Applied Plant Biotechnology laboratory where Australia's collection of banana varieties are maintained *in vitro,* safe entry of imported banana varieties is facilitated and scientists liaise with banana growers, researchers and biosecurity officers from around Queensland and Australia as well as internationally.

Advice and technical support is also provided to the Accredited Quality Banana Approved Nursery (QBAN) laboratories and nurseries in Queensland and NSW to help provide a reliable source of planting material to the grower. Disease free plants rely on virus testing provided by virologists located at DPI&F

Indooroopilly who also work closely with staff at the DPI&F post entry quarantine facility at Eagle Farm to evaluate banana varieties imported into Australia. Advice and technical support is provided to all plant health officers based in banana growing regions in Queensland. The project is geared to respond to disease incursions and to provide varieties for disease exclusion strategies or research. These activities and more have significant positive impact to whole of industry.

To provide Australia with access to the banana germplasm needed to facilitate research and for industry development this project maintains AQIS registration of the Australian banana importation facility to allow industry to gain access to valuable varieties via safe entry of banana germplasm into Australia.

It also importantly maintains a virus indexed banana variety collection *in vitro* of the Australian banana biodiversity which is currently a collection of approximately 4-500 accessions sourced specifically for Australian research. To reduce costs the cultures are stored under reduced growth conditions of low temperature and light. Each culture requires culture onto new media every 6 to 12 months. To maintain quality such as vigorous and true-to-type cultures must be periodically reinitiated. These plants are made available for Australian research aimed at identifying improved varieties from a sustainability, production and market driven aspect. We also maintain and provide disease resistant varieties needed in disease exclusion strategies "on call" and as needed within the "Banana Industry Biosecurity Plan".

Contributing to germplasm maintenance , research and industry development is a field collection containing a subset of banana germplasm in North Queensland. To capture data under commercial conditions and to provide grower access and extension, this project supports a field collection of banana varieties in the tropical North at South Johnstone Research Station. It also provides access to true-to-type suckers to ensure that the tissue culture collection to remain vigorous and true-to-type through culture re-initiation.

The activities in this project have:
· allowed industry to safely import valuable banana varieties
· provided access to Australia's banana biodiversity as a disease free collection of plants
· maintained the level of international biosecurity – in combination with virology research
· supplied disease resistant varieties as part of the disease exclusion or eradication strategy.
·provided disease-free banana varieties for research and industry evaluation.
·Maintained high standards for Australian domestic quarantine. Australia has the best disease free planting material scheme in the world based on accredited commercial tissue culture laboratories and nurseries (QBAN) using tissue culture to produce virus free plants.
·encouraged use of banana tissue culture for improved production and farm management to reduce environmental impacts

Introduction

This project aims to provide long term strategic support in the following main areas underpinned by carefully targeted research to improve the utilisation of tissue cultured plants. The banana industry strategic plan list key priorities described below.

The activities undertaken in this project maintain Australia's banana biosecurity at several levels. By maintaining AQIS registration and contributing to world's best quarantine practice, at both international and domestic level, we allow industry to safeguard entry of valuable new banana varieties into Australia. Industry recognise as a strategic priority the need to minimise future likelihood of pest and disease incursion. Preventing entry of exotic pest and disease while facilitating introduction of disease resistant varieties and preventing movement of pests and diseases within Australia is essential. This role forms an important part of the "Plant Health Australia" National Banana Industry Biosecurity plan.

A wide range of valuable banana varieties has been imported over many years to support Australia's banana research and development. These varieties are continually used to address current strategic priorities such as research into maintaining an environmentally sustainable and viable industry and, in future, the need to anticipate and meet emerging consumer requirements from bananas, in whatever form. Nearly all Australian research into the improvement of banana products, productivity, markets and biosecurity have relied on banana germplasm sourced from the Australian tissue culture collection over the past eighteen years. Indeed the suite of current Australian research programs (eg. virology, NT-Tropical Fusarium race 4 screening, Molecular identification of banana defence genes, soil and root health, leaf and corm diseases, disease exclusion) and industry development programs depend on continued access to a wide range of disease free banana varieties.

A important factor in our sustainability both from production and environment and also global market forces is more than ever before the need to maintain our high levels of biosecurity vigilance to keep exotic disease and pest out of Australia. Continued freedom in Australia from introduced pests and diseases will allow us to provide lower environmental impacts, and better farm labour management, reduced costs and a more marketable product. One important long term aspect will be to find better ways to utilise plant tissue culture to develop improved practices. Australia needs to develop and expand markets using new varieties.

In addition to disease freedom, banana tissue cultured plants also offer production advantages, increased yields and uniformity. However banana tissue culture also has the problem of somaclonal variation "off-types". Research has allowed certain off-types to be removed at nursery phase and developed management strategies that allow tissue culture laboratories to reduce off-types. However off-types, while manageable, still increase costs and reduced grower confidence in the product. Banana off-types cannot yet be identified in culture and mechanisms of somaclonal variation are not understood. To improve sustainability, biosecurity and productivity there needs to be greater use of tissue cultured planting material.

Industry recognise that there is a need to identify and evaluate best practice tissue culture methods with a view to reduction in off types and lower plant cost. To reduce off-types we need to understand what causes them so that we can control their occurrence.

High priorities for the Australian banana industry are to identify export markets and improve production systems. Specific varieties may be needed to satisfy export markets. Plant tissue culture has been used in Australia to prevent the movement of pest and disease. The Australian QBAN scheme (Quality Banana Approved Nursery) is world best practice for delivery of clean planting material. Plant tissue culture now offers growers access to a wide range of these varieties to facilitate niche market development.

Sustainable production practices and market expansion are needed to allow us to remain competitive and will be achieved by a range of approaches. For example we need to find better ways to utilise plant tissue culture to develop improved practices. There are several problematic areas in banana tissue culture to be addressed. Bacterial contamination adds to production costs, compromises quality and reliability of plants reaching the grower. This project uses tissue culture and molecular biology approaches to investigate problems hindering production of banana plantlets including preliminary research investigating possible ways to identify banana off-types *in vitro*.

The Australian banana industry is entering a new era based on the demands of the consumer. Food quality, environmental impacts, combined with increasing pest and disease pressure and increasing global competition are key issues. This project has provided the essential activities needed to implement the banana strategic plan including the biosecurity strategy as well as contributing to an ongoing research effort directed at longer term sustainability issues considering, pests, diseases and markets.

The Australian *in vitro* banana germplasm collection: maintenance and supply.

Introduction:

To support research and industry development, hundreds of banana varieties have been safely imported into Australia in tissue culture through our AQIS registered, Applied Biotechnology laboratory at Nambour. This project is needed to maintain the Australian banana germplasm collection and to facilitate ongoing access to new varieties for research. Australian researchers need to have access to the Australian collection to look for valuable traits such as pest and disease resistance, improved productivity, including more efficient nutrient use for lower environment impacts for less cost. As an example Black Sigatoka resistant varieties have been used for many years in a Northern buffer to prevent Black sigatoka from becoming established in commercial zones. Although the Black Sigatoka was detected in Tully Valley, it has been more than 20 years since the first confirmed Black Sigatoka detection in Australia. Each time we have been able to eradicate and replace susceptible varieties with resistant plants to provide bananas to affected communities in these areas and to encourage cooperation. Disease problems can unfortunately arise unexpectedly and we need to be armed for the response. A current example is the screening of varieties for resistance to Tropical Race 4 Panama in the Northern Territory. These screening programs rely on our arsenal of banana biodiversity while at the same time relying on our ability to import valuable varieties from around the world to solve our problems.

The Australian banana germplasm collection is maintained safely as virus indexed tissue culture. We need to use tissue culture to safely introduce banana varieties into Australia. We need to maintain cultivars free from pests and diseases and can do this providing we initiate cultures only from virus indexed suckers collected from "clean" zones and this allows growers in all quarantine zones to access clean plants. In Australia there are serious diseases in several main production areas in Queensland, Northern Territory and New South Wales. To prevent movement of pests or disease these states are divided into various quarantine zones as part of an important domestic biosecurity strategy to reduce the impact of these pathogens. We also need to safely store our collection away from pests and diseases and severe weather events that can destroy entire collections, as seen recently with cyclone Larry. This project maintains a living collection of approximately 400 accessions in tissue culture to support the Australian banana industry priorities for research and biosecurity. This unique collection has been sourced to meet Australian needs and is recognised internationally as an important banana collection in the Pacific region. This is an active collection not a conservation collection and is continually accessed for research, biosecurity and industry.

Materials and Methods:

In all methods used to initiate cultures, a block of meristematic tissue of approximately 1 cm x 2 cm was excised and sterilized in 2.5 % v/v sodium hypochlorite with Tween 80 surfactant for 15 min. The bleached tissue was then

removed and the resulting block was again sterilized in the bleach solution for a further 5 min, and finally rinsed three times with sterile distilled water.
In the past, plants have been initiated by using shoot-tip culture as described by Hamill *et al.* 1993, but since 1999 all accessions have been initiated into culture using meristem culture to reduce the incidence of bacterial contamination (Hamill and Smith 1999). Explants were initiated into tissue culture following the procedure described by Hamill et at 1993. by meristem culture extracting only the apical dome on a small amount of basal corm tissue covered by approximately three ensheathing leaf bases. Murashige and Skoog media (Murashige and Skoog 1962) modified by the addition of sucrose 20 g l^{-1}, and benzyl amino purine, 2.5 mg l^{-1}, solidified with Phytagel 2.8 g l^{-1}pH adjusted to 6.0 prior to autoclaving at 121°C for 20 minutes was used in all stages of plant tissue culture.

For meristem culture, the meristem (consisting of the apical dome covered by approximately three ensheathing leaf bases) was carefully excised using a dissecting microscope until it was less than 1 mm in diameter and placed directly into media. Meristems were initially cultured in darkness for 5 days before being exposed to fluorescent lighting. All cultures were incubated at 28 °C under GE Polylux fluorescent lights on a 10 hours light, 14 hours dark cycle. Plants were subcultured at four weekly intervals into 125ml glass culture vessels containing 25 ml of the media described above. At the time of culture initiation all individual suckers are screened for bacteria. This method is described in a later section of the document. Using meristem culture combined with bacterial indexing has eliminated bacterial contamination from new accessions and significantly reduced remanent bacteria in the collection.

Tissue cultured banana plantlets in the main germplasm collection are stored under reduced growth conditions to reduce the subculture frequency to between 6 and 12 months (micropropagated banana plants cultured under normal growing temperatures of 28^0C require subculturing every 4-6 weeks). Plants are stored at 16^0C under low light. 20 x 30 ml tubes, each containing one explant, are stored per accession. The banana plantlets are micropropagated as described by Hamill et al. 1992. The media used is Murashige and Skoog (1962) containing 2.5mg/L Benzyl amino purine, 1mg/L Indole Acetic Acid with 20g/L sucrose. When the plantlets require subculturing, either because they need rejuvenating or because they have been requested, half of the plants per accession are taken from the reduced growth main collection and grown at 28^0C to be multiplied until the number of plants required have been produced plus 20 vigorous plantlets for reinstatement into the germplasm collection. Plants to be sent away are placed into standard Murashige and Skoog (1962) media without growth regulators to induce a well developed root system. Depending on the quantity of plants requested, plantlets are sent in sterile 30ml tubes, 250 ml plastic take away food containers.

The *in vitro* collection is supplemented by a field bank at South Johnstone, North Queensland. Tissue culture collections are subject to somaclonal variation, contamination and loss of vigour over time. Accessions are regularly reinitiated into the collection from known true-to-type parent plants sourced from this field collection. Sword suckers of approximately 12cm diameter are carefully collected from true-to-type plants. All suckers are cleaned to remove excess soil and roots pared back to the corm surface using a sharp clean knife. Tops of each sucker are sent to the virus indexing by DPI&F virology group that are part of this project. Each individual sucker was tested for Cucumber Mosaic Virus, Banana Bunchy Top Virus and Banana Streak Virus and Banana Mild Mosaic Virus, details of the virus indexing methods will be described in a dedicated section of this document. All bases of each sucker containing the meristems were sent to the Maroochy Research Station, Applied Plant Biotechnology laboratory for culture initiation as previously described.

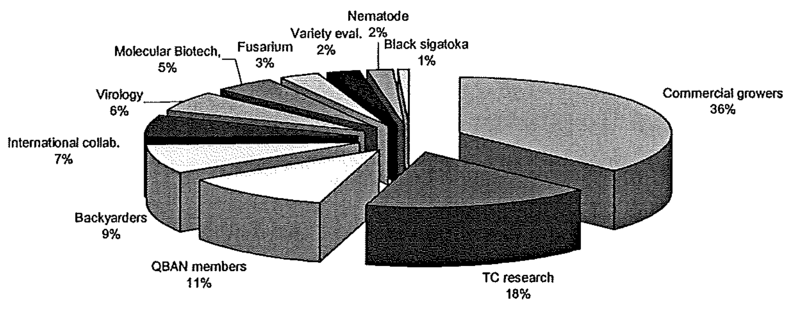
Results and Discussion

One of the main objectives of maintaining the Australian banana germplasm collection is to ensure that we have the diversity of cultivars needed to satisfy industry research and development needs and to make sure that these cultivars can be readily obtained as needed. The extent that cultivars are requested depends on industry buoyancy and direction which influences the level of research and development being undertaken at the time. This project supplied plants that are not available from commercial sources, large quantities of commercial varieties are supplied by accredited members of the Quality Banana Approved Nursery scheme based on virus indexed tissue cultured banana plants.

The banana germplasm collection is a tissue culture collection that is actively used to support research and development and should not be considered a conservation collection. The number and selection of cultivars continually changes due to changes in research needs and future focus. The number of accessions held is limited and approximately 300-500 accessions can be managed within resource constraints. At the end of this project 385 accessions were being maintained in tissue culture.

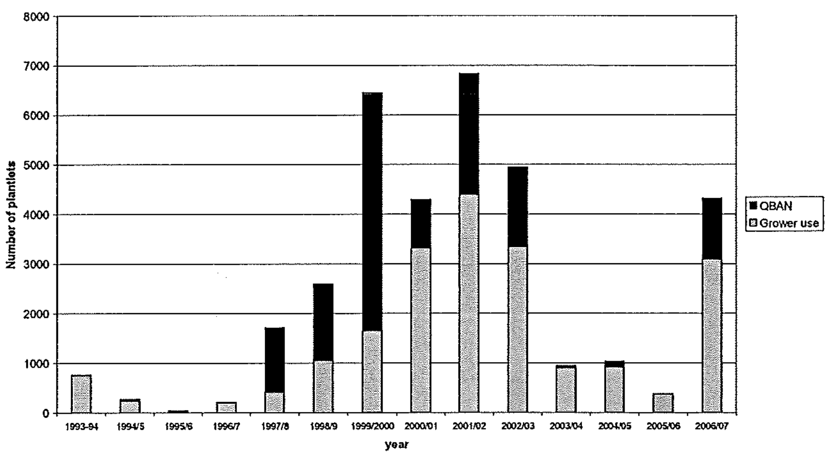
During the three years of this project approximately 12,000 plantlets were provided to support industry in many ways. The distribution of requested germplasm is described in figure 1.

Fig. 1 Distribution of banana germplasm 2004 -2007



A close parallel to the previous three year period was seen regarding commercial requests. In fact 47% of plants produced went to growers who wanted to evaluate new varieties, while starting clean blocks. Figure 2 below describes the supply of banana germplasm for the past thirteen years. There can be seen a general decline in demand for plants between 2003 to 2006, and this was also a period of instability in the banana industry. Low grower returns, threat of imports, no levy to support research was reflected by grower unwillingness to expand at that time. Since cyclone Larry and the good response resulting in a national levy there have been a resurgence in demand from growers. Growers now recognise the value of tissue culture generally and the trends in supply from the commercial Quality Banana Approved Nursery sector are described later in this document. Since the early 2001 commercial QBAN members have improved the quality of plants by restricting multiplication per sucker. Growers are accepting that they must order plants ahead and there is an improved industry self sufficiency regarding supply of tissue cultured plants. This has been a successful culmination of activities in this project.

Figure 2. Commercial Industry demand 1993-2007

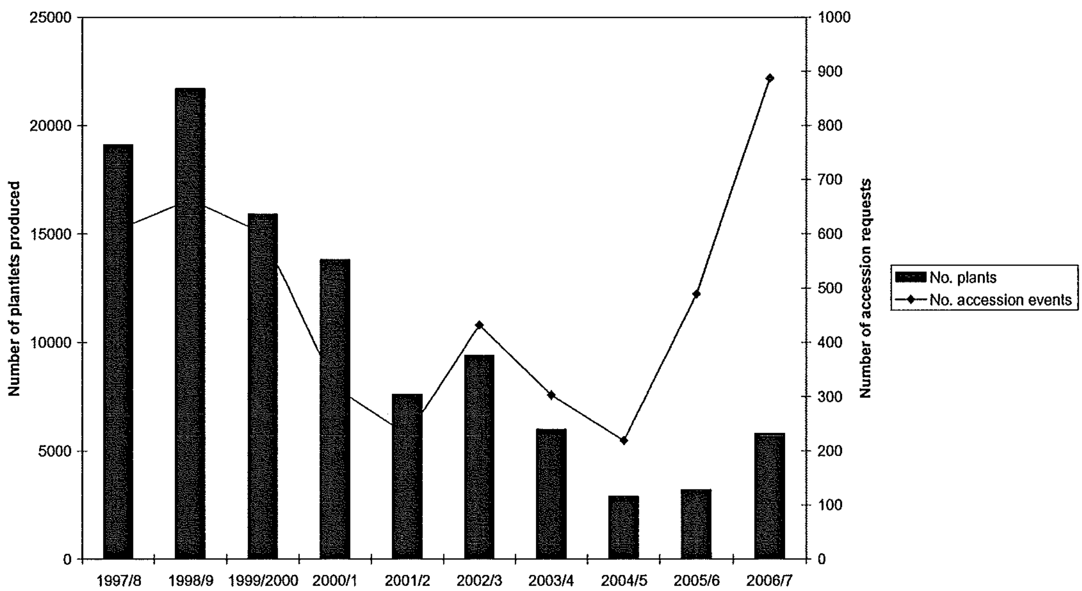


There was a large increase in demand from backyarders wanting bananas after cyclone Larry when banana were scarce (refer fig. 2) . In Queensland backyard growers have limited access to black Sigatoka resistant varieties (in SEQ they can also have lady finger) with up to 10 plants per residence. 9% of plants produced went to backyarders. Most of the backyard demand occurred in 2006/7, largely as a consequence to limited supply of banana fruit after cyclone Larry. While backyard growers should not be encouraged, it is important that clean & resistant plants be able to be readily accessed as an important biosecurity activity to prevent movement of infected suckers across quarantine zones. In addition to the response from cyclone Larry there also appears to be a growing public interest in eating a diversity of types of banana fruit including the cooking types. The aim of future projects will be to assist industry to become self sufficient also in supply to the backyarders as well as small growers. This will be important to maintain good domestic quarantine and to be proactive in supporting industy development and biosecurity activities.

When accessions are requested they are typically taken from the main collection multiplied up and plants replaced in the collection so that there are approximately 20 plants per accession to source plants from and safeguard against loss. Over the past three years these accessions were accessed 1561 times to produce the 12,000 plants required by industry. It must be appreciated that this is an extremely labour intensive activity because on top of the actual tissue culture work there is also a biosecurity component to ensure growers are supplied with information, have been issued associated approved permits, as well as the package and delivery of individual consignments.

Figure 3. describes the trends in demand for plants over the past ten years. While actual number of plants requested has declined, there has been an increase in the number of accession requested at each time. This means there has been a demand in obtaining a wider range of cultivars than seen in previous years and that supports the observation that there is a growing consumer interest in trying a wider range of different types of banana fruits and markets are now embracing the functional food ethos.

Fig. 3 Trends on germplasm demand



An important aspect of maintaining a tissue culture germplasm collection is to ensure the quality of the accessions. There are issues with bacterial contamination and steps developed to overcome are discussed later in the document. It is essential that steps are also in place to reduce operator error such as mislabelling. Another consideration when maintaining tissue culture in the medium to long term is somaclonal variation. Somaclonal variation occurs at a relatively high frequency during the tissue culture process and in general terms results in mutations and epigenetic variations. This variation is not wanted in a germplasm collection where plants are required to be true-to-type representations of their cultivar. To address this issue the *in vitro* germplasm bank is supported by a sub-set of cultivars grown in a field collection at South Johnstone Research Station, Innisfail, North Queensland. To provide and maintain the Australian collection of plantlets in good condition and true-to-type, cultivars are reinitiated into the collection when possible an within resource constraints. Table 1. describes accessions initiated into the collection since the start of this project. When certain cultivars are requested on a regular basis, such as black Sigatoka resistant varieties, these cultivars are reinitiated on a more frequent basis.

Table 1. Varieties initiated into tissue culture 2004 to 2007

Accession Number	Cultivar	Register Number	Date of Receipt
1400	JD Dwarf	M5062	27/10/2004
1401	JD Special	M5063	27/10/2004
1406	Dwarf Red Dacca	M5068	27/10/2004
1409	Williams	M5073	22/11/2004
1410	Williams Dwarf Offtype	M5074	22/11/2004
1417	Sucrier	M5081	22/11/2004
1418	Bluggoe	M5082	22/11/2004
1419	Pisang mas	M5071	22/11/2004
1420	Horn plantain	M5072	22/11/2004
1421	Yesing	M5108	10/03/2005
1422	Taweyawa	M5109	10/03/2005
1423	CJ19	M5110	10/03/2005
1424	CJ19	M5111	10/03/2005
1425	Sar	M5112	10/03/2005
1426	Utufun	M5113	10/03/2005
1427	Kaweputa	M5114	10/03/2005
1428	Menei	M5115	10/03/2005
1429	FHIA 25	M5116	23/03/2005
1430	M.a. malaccensis 845	M5117	23/03/2005
1431	M.a. malaccensis 846	M5118	23/03/2005
1432	M.a. malaccensis 848	M5119	23/03/2005
1433	M.a. malaccensis 850	M5120	23/03/2005
1434	M.a. malaccensis 851	M5121	23/03/2005
1435	M.a. malaccensis 852	M5122	23/03/2005
1436	Dwarf French Plantain	M5123	23/03/2005
1437	Pacifc Plantain	M5124	23/03/2005
1438	Bobby Tannap	M5125	23/03/2005
1439	FHIA 05	M5126	23/03/2005
1440	Lady finger	M5157	28/07/2005
1441	Lady finger	M5158	28/07/2005
1442	Lady finger	M5159	28/07/2005
1443	Lady finger	M5160	28/07/2005
1444	Lady finger	M5161	28/07/2005
1445	High Noon	M5207	28/09/2005
1447	Goldfinger	M5209	28/09/2005
1448	Ducasse	M5210	28/09/2005
1449	Pisang ceylan	M5211	28/09/2005
1450	Pisang kelat	M5212	28/09/2005
1451	Black Ducasse	M5213	28/09/2005
1452	FHIA 26	M5214	28/09/2005
1453	Williams	M5215	28/09/2005
1454	Papat	M5216	28/09/2005
1455	Ducasse	C5296	24/11/2005
1456	Blue java	C5297	24/11/2005
1457	Horn Plantain	C5298	24/11/2005

Accession Number	Cultivar	Register Number	Date of Receipt
1458	Goldfinger	C5299	24/11/2005
1459	Improved Lady Finger	C5300	24/11/2005
1460	Kluai Nawa Khom	C5301	24/11/2005
1461	Buccaneer	C5302	24/11/2005
1462	Calcutta	C5303	24/11/2005
1463	Williams	C5304	24/11/2005
1464	Yangambi	C5305	25/01/2006
1465	PC.1205	C5306	25/01/2006
1467	Pisang ceylan	C5308	25/01/2006
1468	Ducasse	C5309	25/01/2006
1469	Goldfinger	C5310	25/01/2006
1470	Kluai namwa khom	C5311	25/01/2006
1471	M48	C5312	25/01/2006
1472	Pa Payang	C5313	25/01/2006
1473	PKZ	C5314	25/01/2006
1474	PA.1203	C5315	25/01/2006
1475	M.a ssp. Malacensis cross	embryo	6/03/2006
1476	M.a ssp. Malacensis cross	embryo	6/03/2006
1481	D5	M5465	12/07/2006
1482	JD Yangambi	M5466	12/07/2006
1483	Horn plantain	M5467	12/07/2006
1484	Ducasse	M5468	12/07/2006
1485	Blue Java	M5469	12/07/2006
1486	Willams-virus	-virus	12/07/2006
1487	Dwarf Ducasse	M5471	12/07/2006
1488	Lady finger	M5472	12/07/2006
1489	Goldfinger	M5473	12/07/2006
1491	Williams	c5475	20/07/2006
1492	Williams	c5479	20/07/2006
1493	Williams	c5494	14/08/2006
1494	Formosana	M5499	18/08/2006
1495	DPM 25	M5500	18/08/2006
1496	DPM 25	M5501	18/08/2006
1497	DPM 25	M5502	18/08/2006
1498	Goldfinger	M5503	18/08/2006
1499	Ladyfinger	M5504	18/08/2006
1500	Dwarf Cavendish	M5505	18/08/2006
1501	Williams	M5506	18/08/2006
1505	Williams	C5565	27/10/2006
1506	Williams	C5566	27/10/2006
1507	Williams	C5567	27/10/2006
1508	Williams	C5568	27/10/2006
1509	Williams	C5569	27/10/2006
1510	Williams	C5581	6/11/2006
1511	Williams	C5582	6/11/2006
1512	Williams	C5583	6/11/2006
1513	Williams	C5584	6/11/2006

Accession Number	Cultivar	Register Number	Date of Receipt
1514	Williams	C5585	6/11/2006
1515	Williams	C5586	6/11/2006
1516	Williams	C5575	10/11/2006
1517	Williams	C5576	10/11/2006
1518	Williams	C5577	10/11/2006
1519	Williams	C5578	10/11/2006
1520	Williams	C5579	10/11/2006
1521	Williams	C5560	10/11/2006
1522	Formosana	M5592	15/11/2006
1523	Williams	M5593	15/11/2006
1524	Grande naine	M5594	15/11/2006
1525	Ladyfinger	M5595	15/11/2006
1526	PKZ	M5596	15/11/2006
1527	D5	M5597	15/11/2006
1528	Gros Michel	M5598	15/11/2006
1530	DPM 25	M5600	15/11/2006
1531	DPM 25	M5601	15/11/2006
1532	R553	M5602	15/11/2006
1533	DPM 25	M5603	15/11/2006
1534	Horn Plantain	M5604	15/11/2006
1535	Williams	C5605	20/11/2006
1536	Williams	C5606	20/11/2006
1537	Williams	C5607	20/11/2006
1538	Williams	C5608	20/11/2006
1539	Williams	C5609	20/11/2006
1540	Williams	C5610	20/11/2006
1541	Williams	C5616	27/11/2006
1542	Williams	C5617	27/11/2006
1543	Williams	C5618	27/11/2006
1544	Williams	C5619	27/11/2006
1545	Williams	C5620	27/11/2006
1546	Williams	C5621	27/11/2006
1547	Williams	C5627	1/12/2006
1548	Williams	C5628	1/12/2006
1549	Williams	C5629	1/12/2006
1550	Williams	C5630	1/12/2006
1551	Williams	C5631	1/12/2006
1552	Williams	C5632	1/12/2006
1553	Kluai Namwa khom	M5649	21/02/2007
1554	Goldfinger	C5650	21/02/2007
1555	Ducasse	M5651	21/02/2007
1556	Pisang ceylan	M5652	21/02/2007
1557	Lady finger	M5653	21/02/2007
1558	Bluggoe		21/02/2007
1559	Blue java	M5655	21/02/2007
1560	Williams	C5656	21/02/2007

Accession Number	Cultivar	Register Number	Date of Receipt
1564	Williams	C5657	28/03/2007
1565	Williams	C5658	28/03/2007
1566	Williams	C5659	28/03/2007
1567	Williams	C5660	28/03/2007
1568	Williams	C5661	28/03/2007
1569	Williams	C5685	30/03/2007
1570	Williams	C5686	30/03/2007
1571	Williams	C5687	30/03/2007
1572	Williams	C5688	30/03/2007
1573	Williams	C5689	30/03/2007
1574	Gros Michel	C5708	16/05/2007
1575	Lady Finger	M5709	16/05/2007
1576	Williams	C5710	16/05/2007
1579	Senorita	M5713	16/05/2007
1580	Goldfinger	M5714	16/05/2007

Importation of new varieties

Introduction

Facilitation of safe importation of banana cultivars into Australia is an important activity provided by this project. Banana cultivars can only be imported into Australia as tissue cultured plantlets accompanied by the appropriate phytosanitary certification. Further post entry screening occurs and takes up to two years to complete. To undertake the stringent steps required to ensure the tissue culture plants are not carrying pathogens including and especially virus, AQIS registration of a tissue culture laboratory has been maintained in this project for importation of tissue culture stock. It is a critical biosecurity step in Australia's quarantine to maintain a system to keep Australia free of exotic pests and pathogens found in other countries.

Materials and Methods

Banana plants can only be introduced into Australia as tissue culture. The cultures are established as individual growing points. Each of these cultures is multiplied to 10 plants and the leaves are taken aseptically and sent for virus testing. All imported banana tissue cultured plantlets must be tested at a very early stage (when the plantlets are still in tissue culture containers) by using reliable serological (including immuno-sorbent electron microscope (ISEM)) and/or molecular tests for ensuring freedom from banana bunchy top virus (BBTV), banana bract mosaic virus (BBMV), banana streak virus (BSV) and undetermined rod shaped viruses. A single meristem from a tissue cultured plantelet is multiplied up to 10 plants and from this a representative sample of a minimum of three plants from each accession (i.e. each tissue culture container) must be grown in post-entry quarantine until they have reached a 10 leaf stage and tested for freedom from BBTV, BBMV, BSV and undetermined rod shaped viruses using effective serological (including ISEM) and/or molecular tests. Additional tests will be conducted if and when virus symptoms are detected during the intermittent period. After drawing a sample from an accession for growth in post-entry quarantine, the remaining tissue cultures may be multiplied in an AQIS-approved laboratory. All multiplied plants/plantlets derived from imported materials must remain in quarantine until a representative sample has completed all post-entry quarantine requirements.
All plant material including tissue cultures must be sufficiently labelled and identified so that AQIS is able to trace the fate of consignments.

All precautions must be taken to prevent cross infection of plants and tissue cultures during propagation and collection of samples for disease diagnosis. Cutting tools are disinfected before each use (ie between plants and corms) by dipping in alcohol and flaming for a few seconds. Alternatively, disposable blades may be used and discarded after each use.

Any plant(s) which dies in post-entry quarantine, together with associated potting medium, is to be autoclaved (121°C for 30 mins) or disposed of by an AQIS approved method. All used pots are to be thoroughly washed of all potting medium and root debris in a sink which drains into a sewerage system and then sterilised.

Upon completion of post-entry quarantine, with no detection of quarantinable diseases, plants in post-entry quarantine and, if applicable, related tissue cultured plants (multiplied in quarantine) may be released from quarantine subject to approval of an authorised AQIS officer.

If a quarantinable disease is intercepted in post-entry quarantine, the Chief Quarantine Officer (Plants) is to be notified immediately. The Chief Quarantine Officer (Plants) will in turn notify AQIS, Canberra. Plants, plant parts and tissue cultures found to be infected with a quarantinable disease are to be destroyed by an AQIS approved method or may be re-exported under secure quarantine conditions.

Banana plants will not normally be permitted entry into Australia. All applications to import banana plants or plant parts such as corms, suckers and bits must be referred to Plant Programs Section in Central Office. If special circumstances warrant the introduction of plants or plant parts the following conditions will apply:

The importer must possess a valid import permit. Permit applications may be approved by the States. Plants or plant parts may only be imported for the purpose of generating tissue cultures in an AQIS-approved laboratory. Material must be bare rooted and free from soil, disease symptoms and other extraneous contamination. Material must be packed in new containers and must be labelled with scientific names.

Material is subject to inspection on arrival for freedom from pests and diseases, soil and other extraneous matter. If a quarantine disease is detected the infected material is to be destroyed or may be re-exported.

Original packaging material is to be incinerated.

Fumigation of imported material is <u>not required</u> providing all material is kept under secure conditions and autoclaved immediately after collecting explants (meristems) for tissue culturing in an AQIS-approved laboratory.

All banana introductions are to be tissue cultured in an AQIS-approved laboratory equipped with appropriate facilities to handle, treat and dispose of imported materials and derivatives thereof in a safe way. After establishing tissue cultures, residual plant material must be autoclaved (121°C for 30 mins) or destroyed by an AQIS-approved method.

Results & discussion

AQIS registration was maintained over the period of the project to facilitate entry and evaluation of banana tissue cultured plants. Registration requiring six monthly audits and annual renewal of premises as well as AQIS quarantine training for staff. The following accessions (refer table below) went through the quarantine system between 2004-2007, with many now released. Cultivars imported this year will be in quarantine for another year or so.

Accessions undertaking quarantine post entry screening 2004-2007

Accession Number	Cultivar	Collection Location	Date of Receipt
1246	Cardaba	Belgium	15/04/2002
1248	PA12.03	Belgium	15/04/2002
1249	PV42-53	Belgium	15/04/2002
1250	PV42-81	Belgium	15/04/2002
1251	PV42-320	Belgium	15/04/2002
1252	JV42-41	Belgium	15/04/2002
1253	B 7925	Belgium	15/04/2002
1255	TMB3x15108-6	Belgium	15/04/2002
1294	Taweyawa	Papua New Guinea	12/09/2002
1357	Utin lap	Micronesia	12/06/2003
1362	Karat	Micronesia	12/06/2003
1364	Mangat	Micronesia	12/06/2003
1355	Ipali	Micronesia	12/06/2003
1561	Grower import	Philippines	25/01/2007
1562	Grower import	Philippines	25/01/2007
1563	Grower import	Philippines	25/01/2007

There is ongoing and sporadic need for new banana cultivars into Australia. Usually the need for banana germplasm relates to the amount of banana research activity being supported in Australia. In recent years the amount of banana related research has been low compared to previous times and this has been reflected in quantities of germplasm imported for evaluation. However whether there are large or small quantities of banana material coming into Australia it is equally important that we maintain a high quarantine standards to facilitate safe access to imported plants. The facility and expertise supported in this project projects Australia with the ability to safe access to improved varieties without compromising our International Biosecurity status.

Banana Field germplasm collection and agronomic evaluation of new varieties

Introduction

Bananas are Australia's most important fruit crop with the gross industry value in excess of $390 million in 2007 (DPI&F 2007). Production is mostly of Cavendish cultivars but with significant area of Lady Finger and a handful of niche market varieties.

A large collection of banana varieties has been maintained in the field at South Johnstone Research Station and *in vitro* at Maroochy Research Station, Nambour Queensland since the late 1980's to support biosecurity activities such as the black Sigatoka disease buffer zone in Cape York/Torres Strait, to support our international research projects in the Asia/Pacific region and Australia all of which has contributed to the Department of Primary Industries and Fisheries high international standing in banana research and development. As well, planting material has been provided to growers wishing to trial new varieties.

The banana industry is faced with many problems and opportunities so we are constantly on the look out for pest & disease resistant varieties, higher yielding lines or those that might offer some other advantage in the market place.

We have an ongoing introduction program where promising varieties from overseas are obtained for evaluation. These come from various breeding programs and field collections. Additional selections from within Australia are also evaluated.

Materials and Methods

The *in vitro* collection maintains virus indexed plants in a disease-free condition suitable for dissemination whilst the field collection is used for demonstration purposes (on site and for display purposes at events such as banana congresses) and provides suckers for regular re-initiation of tissue cultures to minimize problems associated with development of somatic mutations whilst in culture.

A tissue culture authentication trial was planted in 2005 to determine the extent of somatic mutations in some lines in the Maroochy in vitro collection.

The banana field collection of in excess of 200 varieties is maintained on the South Johnstone Research Station. Each variety is maintained as a single clump containing multiple stems at various stages of development.

The studies were conducted at South Johnstone, north Queensland (17°38'S) on a deep alluvial light clay (inceptisol). Plant spacing was 5 m x 1.5 m (1333 plants/ha). The number of sample plants varied from 2 to 14. When there were sufficient plants a randomized block design was used with 2 replicates.

The trial area received basal lime applications (2 t/ha), which was broadcast and incorporated prior to mounding and drilling out the rows. An NPK blend

(11.1:1.7:17.6) was broadcast in the drill at 500 kg/ha just before planting. Potassium (600 kg/ha/yr as KNO3) plus supplementary nitrogen as urea (total N of 400 kg/ha/yr) was applied in the irrigation water at monthly intervals.

Under tree mini-sprinklers ensured that water was freely available to plants throughout their growth. Weeds, pests and diseases were controlled as per normal industry practice.

The agronomic trials reported on are best described as observational trials – a preliminary assessment only. In many cases insufficient plants were available to permit statistical comparisons but data was obtained to indicate likely usefulness. The varieties came from a range of sources and were mostly introduced to Australia in the project prior to the one reported here (it takes almost 2 years to import and clear quarantine before reaching the field). The following varieties were evaluated between 2004 and 2007 in a set of 5 evaluation trials.

Planting 1	**Planting 2**	**Planting 3**	**Planting 4**	**Planting 5**
Williams	Ducasse	Williams	Williams	Formosana
Novaria G	Black Ducasse	R6	DPM25/ 1334	Williams
Novaria D	FHIA-26	Lady Finger	DPM25/ 1335	Grande Naine
Mutaria	SH-3697	PV 42.81	DPM25/ 1336	
Ducasse x Rodger	PKZ	PV 42.320	'Cardaba'	
Ducasse x Butuhan	D5	JV 42.41	PV 42.53	
	RSS3	PA 12.03	Ouro da Mata	

The varieties were evaluated progressively as they became available. The first planting was established in November 2002, the second in July 2003, the third in March 2004 and the fourth and fifth in June 2004. The trial plantings were established from tissue culture plants provided from the Maroochy laboratory. Plants were grown for a plant and ratoon crop.

Bunches were harvested once per week and consisted of those bunches which had begun to ripen in the field, since little was known about the harvest times for many of the varieties. The characteristics recorded at harvest included bunch fresh weight, finger length and diameter on hand 3, and pseudostem height. However, comparisons among Cavendish cultivars were made at standard commercial harvest (> 3.7 cm diameter hand 3).

Cyclone Larry destroyed the fields on 20 March 2006. Fortunately the damage occurred towards the end of the ratoon cycle for trials so that no entire trial data was lost.

Results & Discussion.

Authentication of plants stored in the medium term *in vitro* collection.

23 varieties of banana plants that had been stored long term in the *in vitro* germplasm collection from the Maroochy laboratory were deflasked and established in the field in 2005 specifically for the purpose of evaluating plants to ascertain if they were true-to-type or contained off-types. All accessions evaluated were true-to-type except one variety ('Ice Cream') an off-type. The off-type was much taller and had a very poor bunch.

Supply of suckers from true-to-type cultivars for reinitation of cultivars to maintain a high quality banana germplasm collection.

Suckers of different varieties were dug for re-initiation of tissue cultures throughout the course of the project. The trimmed and cleaned bases of individual suckers were freighted to Maroochy R. S. for establishment in tissue culture. The tops of individual suckers containing leaf samples needed for virus indexing were sent on each occasion to Indooroopilly DPI&F for virus indexing. Over the three years 673 suckers were harvested on 13 separate occasions, accessing varieties 110 times. Refer table below.

Table 1

Number of varieties	**Number of suckers collected**	**Certificate ID**
10	56	710
10	39	711
2	9	712
11	62	713
10	73	715
9	58	005
11	57	006
10	56	228
9	67	233
3	15	250
10	72	249
8	67	179
7	42	134
110 times varieties accessed	673 suckers	13 collection events

Impact of cyclone Larry on field collection

Cyclone Larry devastated the collection on 20 March 2006. The collection was extensively damaged interrupting and destroying agronomic evaluation blocks established at that time. The collection was cleaned up ,and plants trimmed back so that it regrew satisfactorily as the plants were mostly bent over by the strong winds rather than entirely uprooted.

Variety Observations and agronomic evaluation trials, South Johnstone

The results of the variety observations are presented in Tables 1-5. Photos were taken of mature bunches and these along with agronomic data will be included in the database planned for the next project which continue on from the work in this project.

Planting 1

Novaria D and Novaria G were Malaysian selections of GN-60 A (which had been obtained by irradiating Grande Naine Cavendish). The Novaria selections were supposed to be earlier flowering but there was no indication that this was the case for the few plants that we examined. In fact they appeared to ratoon slower than Williams in our observations. Plants were about 14% shorter than Williams in the plant crop and about 20% in the first ratoon which is relatively standard when Grande Naine is compared to Williams. Novaria was also reported to have some tolerance to Tropical race 4 of Fusarium wilt (TR4 *Foc*) but screening in the Northern Territory obtained a very susceptible reaction for both Novarias.

Mutaria is a selection of Sugar (AAB, Silk) that was reported to have resistance to TR4. The few plants we grew appeared similar in plant characteristics to Sugar. Unfortunately in screening trials in the Northern Territory it too proved very susceptible to TR4. However, it may be worth screening against Race 1 *Foc* at a later date in north Queensland.

We grew on various hybrids that had been generated from some hand pollinations of Ducasse (ABB, Pisang Awak) from studies examining whether banana mild mosaic virus, which commonly infects Ducasse, could be transferred to progeny via true seed. None of the hybrids appear to have any immediate commercial potential but one does have a rather interesting characteristic – it seldom produces following suckers. Perhaps we have stumbled upon a 'terminator' gene which may be useful in future research.

Table 2 Planting 1 of Varieties at South Johnstone

Variety	planting - harvest (days)	bunching - harvest (days)	bunch weight (kg)	hand 3 diameter (cm)	hand 2 length (cm)	finger number /bunch	stem height (cm)
Plant Crop							
Williams	344	126	30.4	3.8	27.2	147	261
Novaria G	338	122	28.6	3.8	26.6	142	225
Novaria D	347	129	30.0	3.8	26.3	148	227
Mutaria	400	128	22.3	4.0	18.5	138	321
Ducasse x Rodger	551	151	21.0	4.0	18.2	173	421
Ducasse x Butuhan	430	130	14.5	3.4	16.1	121	235
Ratoon							
Williams	594	130	36.8	3.6	26.5	214	325
Novaria G	623	136	32.6	3.7	25.9	178	264
Novaria D	676	156	28.8	3.8	24.6	152	253
Mutaria	722	145	20.5	3.5	18.4	166	416
Ducasse x Rodger	866	159	17.0	4.7	18.9	95	503
Ducasse x Butuhan	did not sucker						

Planting 2

SH-3697 is a Maia Maoli/Popoulu hybrid from the Honduran banana breeding program. It is a type of plantain which can produce very large bunches. Unfortunately it is infected with banana streak virus (BSV) so it can not be utilized further. In many cases BSV killed the growing point of plants and caused other bunch and plant abnormalities.

FHIA-26 is a Ducasse hybrid also from Honduras. Unlike so many other hybrids it is remarkably similar to its female parent (Ducasse) in appearance and taste. We had imported it primarily because it was hoped that it would have resistance to Race 1 *Foc* which is a major problem for the production of Ducasse in Australia. However, screening observations on 2 growers' properties in north Queensland indicate that it is susceptible. Nevertheless, FHIA-26 may have some prospects as our preliminary observations have indicated that its bunch weight is about 14% more which was a function of slightly longer and fatter fruit. The next step should be to investigate commercial production in sites free of Fusarium.

In recent years the southern markets have indicated that there is more than one type of Ducasse and that they know the preferred type as 'Black Ducasse'. We obtained tissue culture material of what was said to be Black Ducasse but our observations do not indicate any particular differences.

PKZ is a supposed selection of Goldfinger made in South Africa which is closer in bunch features to Cavendish but retaining the resistance to Subtropical Race 4 of Fusarium wilt. Based on its bunch features I find it very difficult to believe that it is a somatic mutation of Goldfinger. It was imported by Australian Nurseryman's Fruit Improvement Company (ANFIC) who currently have the rights to it in Australia. Though high yielding the fruit flavour was nothing remarkable so is unlikely to have any prospects.

Table 3 Planting 2 of Varieties at South Johnstone

Variety	planting - harvest (days)	bunching - harvest (days)	bunch weight (kg)	hand 3 diameter (cm)	hand 2 length (cm)	finger number /bunch	stem height (cm)
Plant Crop							
Ducasse	470	176	32.2	4.1	19.6	242	421
Black Ducasse	486	175	33.2	4.3	19.0	231	446
FHIA-26	466	169	36.8	4.4	20.3	234	449
SH-3697	498	155	63*	4.7	32.4	249	440
PKZ	441	159	38.0	3.8	23.3	244	337
D5	346	127	36.0	3.7	27.5	174	262
RSS3	339	126	38.0	3.8	27.9	189	280
Ratoon							
Ducasse	670	132	31.8	4.1	20.0	211	436
Black Ducasse	677	130	29.1	4.2	20.0	172	450
FHIA-26	625	118	27.3	4.2	20.9	149	449
SH-3697			**				
PKZ	781	143	40.8	3.8	23.6	258	363
D5	617	97	47.1	3.9	28.5	201	273
RSS3	615	100	50.5	3.8	29.3	241	281

* 90% of plants fell over before harvest;
** no bunches harvested

Planting 3

We evaluated 5 Lady Finger hybrids from EMBRAPA's breeding program in Brazil. PA 12.03 was a dwarf hybrid while the others were much taller, actually 14-24% taller than Lady Finger across the 2 crops. Bunch weights of 3 of the latter hybrids were 16-30% heavier than Lady Finger and fruit were up to 15% longer. Add to this that these hybrids are said to have resistance to Race 1 *Foc* (although testing is required in Australia) and yellow Sigatoka (observations in north Queensland support this) and some have quite good flavour (PV 42.81 and PV 42.320) makes them very interesting.

Alarmingly however, they suffered considerable wind damage in the plant crop (17-86% losses) compared with no losses in Lady Finger. By contrast there were relatively few wind losses in the first ratoon. In many cases the wind loss was unusual with the pseudostem and attached bunch literally snapping off midway up the pseudostem. The dwarf hybrid did not sustain any wind losses but its fruit flavour was disappointing.

TMB 3x15108-6 from the breeding program at IITA in Nigeria was infected with BSV and seldom produced a viable bunch.

We evaluated R6 – a secondary triploid from the Jamaican banana breeding program. The dwarf plant looked very promising but the bunch features and eating quality were inferior.

Table 4. Planting 3 of Varieties at South Johnstone

Variety	planting - harvest (days)	bunching - harvest (days)	bunch weight (kg)	hand 3 diameter (cm)	hand 2 length (cm)	finger number /bunch	stem height (cm)
Plant Crop							
Williams	381b	111d	50.8a	4.05a	28.2a	218a	288d
R6	406b	131b	25.2e	3.33c	26.7ab	144b	256e
Lady Finger	385b	141a	23.5e	4.07a	23.3cd	99d	359c
PV 42.81	441a	137ab	34.5b	3.87ab	24.8bc	149b	472a
PV 42.320	381b	115d	28.0d	3.77b	25.2bc	119c	408b
JV 42.41	377bc	122c	31.6c	4.04a	26.7ab	121c	412b
PA 12.03	349c	125c	19.1f	3.73b	21.9d	102d	238e
Ratoon							
Williams	681	129c	56.1a	4.33ab	28.1a	213a	325cd
R6	689	127c	28.3b	3.39e	25.2ab	175b	291d
Lady Finger	689	169a	28.0b	4.49a	22.4b	113d	458b
PV 42.81	696	146b	31.8b	3.71d	24.3b	141c	543a
PV 42.320	711	133bc	31.6b	3.93cd	23.7b	144c	522a
JV 42.41	722	134bc	35.4b	4.20b	25.3ab	135c	524a
PA 12.03	598	177a	30.5b	4.13bc	23.8b	141c	353c

* means with same subscript are not significantly different at the P = 0.05 level. There were no significant differences in columns without letters.

Planting 4

Cardaba was imported from the International Transit Centre collection in Leuven , Belgium and was supposed to be a Saba cooking banana (the most popular banana in Philippines) but it is incorrectly labelled/classified and has no immediate prospects. Ouro da Mata, a naturally occurring tetraploid, was also infected with BSV.

We compared 3 accessions of a variety developed from Giant Parfitt by mutation breeding applications using gamma irradiation by Smith and Hamill (Smith et.al. 2006), with the industry standard Williams. This cultivar has improved tolerance to subtropical Race 4 Fusarim wilt compared to Williams but has similar yield under subtropical conditions. Yields and plant characteristics were much the same as Williams. Thus there may be an opportunity to deploy them in areas with Subtropical Race 4 *Foc* (for which they have some tolerance) without sacrificing on agronomic qualities as is often the case with *Foc* resistant variants of Cavendish.

Table 5. Planting 4 of Varieties at South Johnstone

Variety	planting - harvest (days)	bunching - harvest (days)	bunch weight (kg)	hand 3 diameter (cm)	hand 2 length (cm)	finger number /bunch	stem height (cm)
Plant Crop							
Williams	360b*	118	38.1	3.76	26.9	180	280
DPM25/1334	343a	116	39.9	3.78	27.1	191	289
DPM25/1335	335a	117	39.6	3.76	27.2	192	284
DPM25/1336	351b	122	39.9	3.74	26.8	193	290
Ratoon							
Williams	622	109	46.3a	3.85	26.4	228	303
DPM25/1334	623	100	45.3a	3.75	27.0	214	295
DPM25/1335	617	100	45.3a	3.73	27.1	231	309
DPM25/1336	610	102	42.3b	3.79	27.7	208	278
Others (plant crop)							
'Cardaba'	404	154	17.1	4.3	18.3	109	365
PV 42.53	425	171	22.3	3.7	22.9	124	442
Ouro da Mata	350	107	24.3	4.4	19	121	409

* means with same subscript are not significantly different at the P = 0.05 level. There were no significant differences in columns without letters.

Planting 5

DPI&F Program Leader, Bob Williams, imported Formosana about 3 years ago from the Taiwan Banana Research Institute. Formosana, which is also known as GCTCV 218 and NC 301, is a tissue culture off-type of a Giant Cavendish selected for resistance to subtropical race 4 of Fusarium wilt (Panama disease). It is now being cultivated commercially in Taiwan.

After Formosana completed its period in quarantine, tissue cultured plantlets were sent to (i) the Northern Territory to be screened for resistance to tropical race 4 Fusarium wilt by NT government staff led by Geoff Walduck (ii) South Johnstone for agronomic evaluation (plant characters/yield etc). Field trials began in January 2004.

Table 6. Plant crop - yield results ratoon data not collected

Variety	Bunch weight (kg)	Months Planting - Harvest	Bunch wt/12 months	Finger length hand 3 (cm)	Pseudostem height (m)
Formosana	44.4	15.9	34.0	27.5	3.24
Williams	35.9	12.4	35.2	27.4	2.46
Grande Naine	32.9	12.1	33.1	26.9	2.32

Larger Bunches but Slower Cycling
Preliminary results from South Johnstone indicate that Formosana took about 3 months longer (12 months vs 9 months) till bunching than Williams/Grande Naine. However, the few bunches of Formosana so far harvested have been in excess of 40 kg – about 10 kg heavier than Williams and Grande Naine. Plants of Formosana were about 70 cm and 90 cm taller than Williams and Grande Naine respectively.

The Tropical Race 4 Threat
Currently this strain of Fusarium wilt is not present in north Queensland but has had devastating effects on the small Cavendish industry near Darwin in recent years. Strict quarantine is crucial for keeping the disease out of north Queensland. However, should a disease incursion occur in north Queensland it would be invaluable to have a resistant Cavendish variety for replanting. Geoff Walduck from the NT will report elsewhere on results of their disease screening but preliminary results are promising with Formosana showing some tolerance to the disease, particularly when disease pressure is not severe.

Recommendations and Future Directions

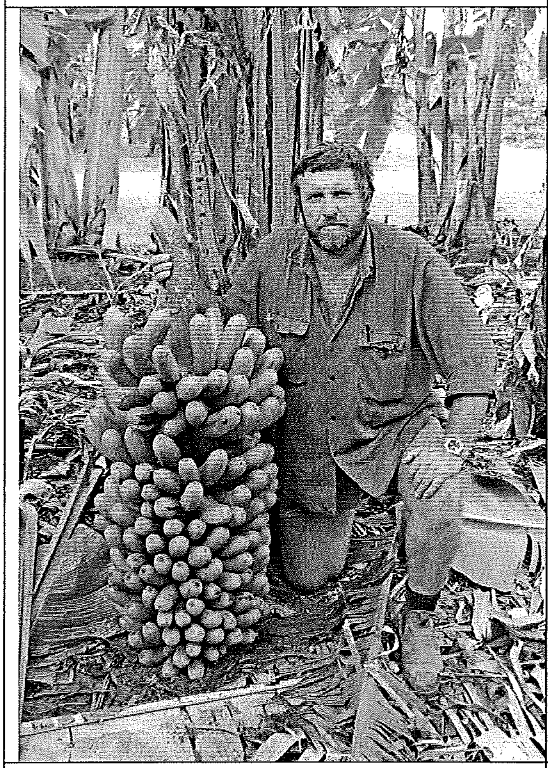
Ownership issues associated with selections from overseas remains a stumbling block to the further development of certain varieties that may benefit the industry. ANFIC/BRI have the rights in Australia although this may over time have elapsed. The Queensland Banana Industry Protection Board convened a meeting of the various stakeholders on 5 December 2001 to chart a way forward but industry preoccupation with the black Sigatoka outbreak in Tully and the fight against Philippine imports plus a general dearth of funds (lack of compulsory industry levy) has largely caused this issue to remain in limbo. Major increase in commercial production of niche market varieties depends on market development initiatives which are beyond the scope of the current work.

There is a lack of consolidated information on agronomic characteristics of bananas in Australia. Future aims in the forthcoming project will be to collate the data and photo set that has been collected over several years into a database. A single database rather than many separate publications and documents will improve access to the information that is needed for decision making by prospective growers of new varieties.

Banana streak virus (BSV) continues to loom as a threat to germplasm resources. Several hybrids obtained from overseas breeding programs have the activated form of BSV present. To make sure our germplasm resource is not jeopardised we intend indexing all varieties in the field collection in the early part of the forthcoming project.

PKZ looks nothing like Goldfinger

FHIA-26 is similar to Ducasse but appears to be higher yielding



63 kg plant crop bunch of SH-3697 but BSV is a major problem

Lady Finger hybrids from Brazil suffered severe wind damage in plant crop

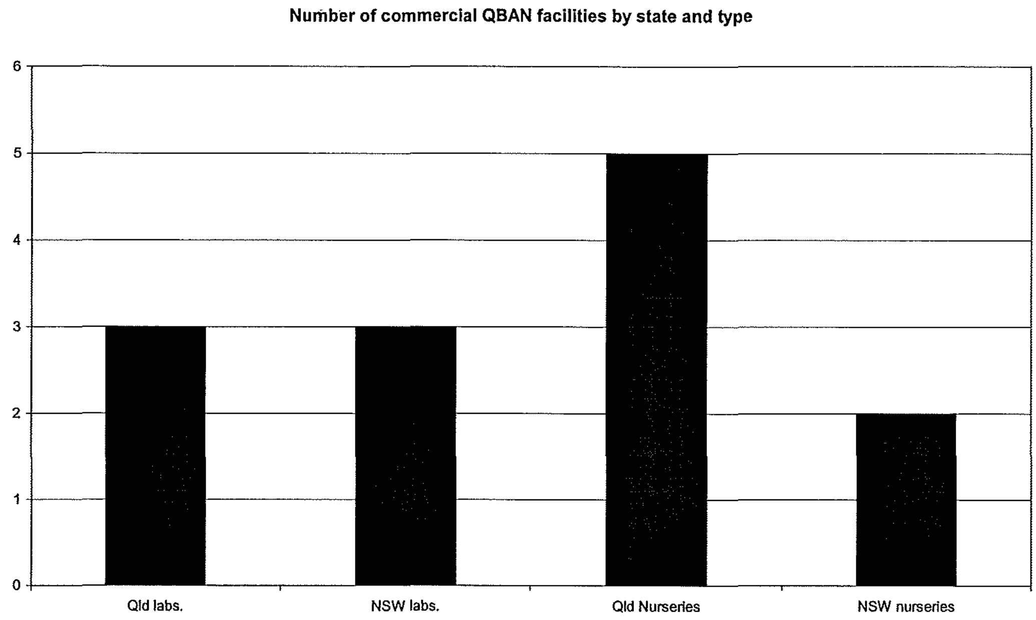
Quality Banana Approved Nursery Scheme (QBAN)

Introduction

The Quality Approved Banana nursery scheme was established in 1994 as a means to reduce spread of pest and diseases in affected zones. The diseases of most impact are Banana Bunchy Top Virus and *Fusarium oxysporum* f.sp. *cubense*. The QBAN scheme relies on establishment and subsequent multiplication of tissue cultured plants from individually virus indexed banana suckers. In addition to freedom from pest and diseases plants produced from tissue culture offer many benefits for improved farm production. Since 1994 industry has been working to encourage uptake of tissue culture for both Biosecurity and farm productivity gains. In Australia there has been a reluctance by growers to change to tissue culture even though farmers around the world given the opportunity made a rapid change to adopt tissue culture. Australian growers generally are not facing the same disease pressures as most commercial banana producing countries and had not adopted tissue culture due to several factors that included change of farm practice, cost, need to order ahead and pay deposit and a previous experience or perception of high levels of off-types. More recently tissue culture has played an important part in improving farm efficiencies particularly important with increased costs of production and ongoing labour shortages. Now there appears to be a growing demand for tissue culture and industry is now faced with the challenge of managing and regulating the growing demands for the QBAN scheme.

Results

There has been consolidation of QBAN registered facilities since the last report was issued in 2004. In 2007 there are currently 13 registered laboratories and nurseries down from 27 registered in 2004. Almost all of these facilities were established when QBAN commenced, although there are several new nurseries in Queensland and more laboratories and nurseries are in the process of being established. QBAN registered tissue culture laboratories can supply banana plants to anywhere in Australia so location is not a restriction and there is an equal distribution of laboratories in Queensland and New South Wales. However nurseries generally are located in the area where the plants are to be grown and must be in a zone free from notifiable pest or disease. Nursery location is influenced by quarantine zone restrictions. Since most of Australia banana production is in North Queensland this is where the majority of nursery plants are produced.



To facilitate research there are also 6 research institutions registered and accredited as QBAN facilities.

QBAN administration

The Administration of QBAN and maintenance of the QBAN database and clone register has been managed by Department of Primary Industries and Fisheries (DPI&F), Queensland Biosecurity staff based at South Johnstone Research Station, Innisfail. Until November 2007 DPI&F plant health officers have inspected suckers destined for tissue culture and supervised their collection as described below.

Financial Year	Suckers collected*	Initiation material inspections	Approvals to move initiation material
04/05	1830	16	21
05/06	2950	32	49
06/07	2830	39	48

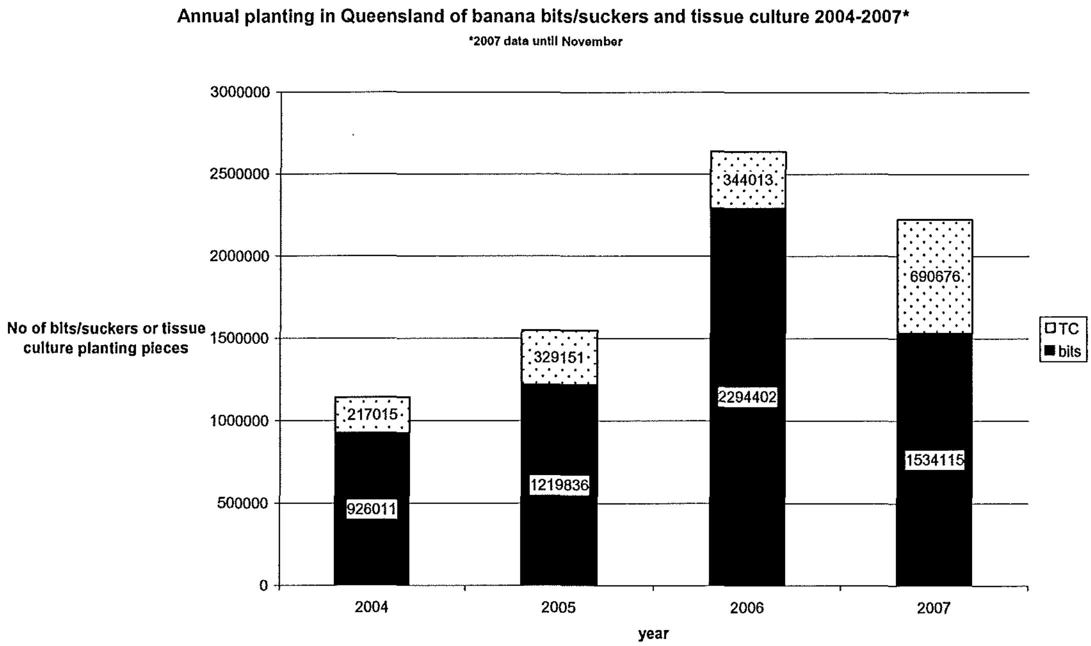
*In a previous report 2005, this figure would have been reduced by a factor of ten as the data provided at that time was related to clone number of the mother plants and not the actual number of suckers, usually ten suckers are collected per mother for virus indexing in batches.

There is an increasing demand for tissue culture particularly as large commercial growers begin to appreciate the improvements to farm efficiency that tissue cultured plants can provide. This increase in demand also translates to more suckers needed and permits issued and Biosecurity staff are finding it difficult and challenging to be able to meet these increasing demands within their current resource constraints.

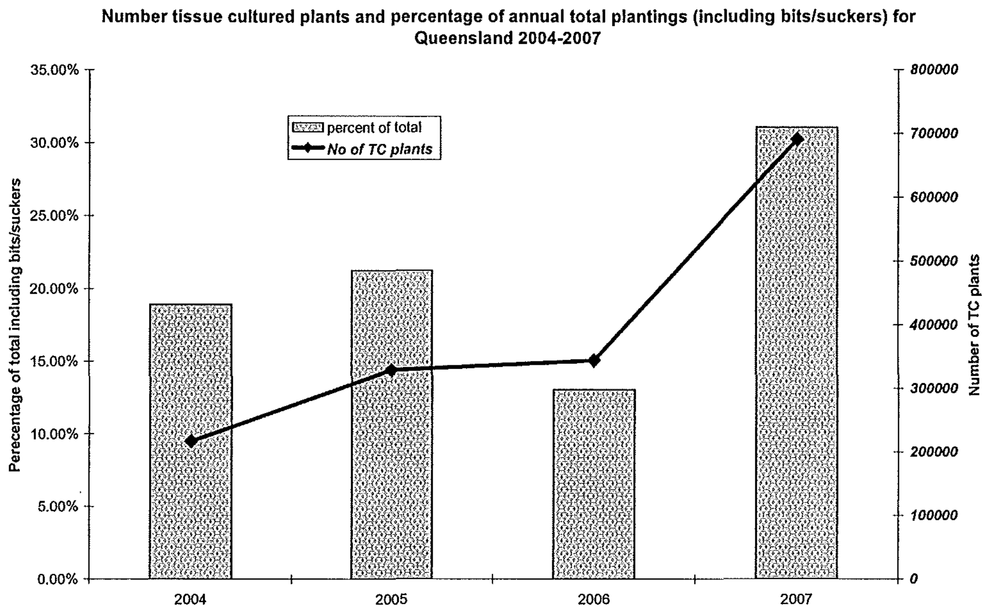
Impacts and changes to QBAN

There have been progressive changes to the way that QBAN is administered and data collected. Originally QBAN was administered by the Secretary if the Queensland Banana Industry Protection Board. The secretary issued clone numbers after virus results found suckers negative to virus and issued permission to move between QBAN facilities in both New South Wales and Queensland. For the past few years the movement of banana tissue culture in sealed inspect proof containers has been regulated by plant health officers in each state rather than by a single regulatory body. Plant health officers in each state issue a plant health certificate to allow movement intrastate after confirming plant have been virus indexed and found to be free from virus. There is then the requirement for growers to obtain permission to plant and cultivate prior to obtaining the tissue cultured plants subject to each states acts and regulations. As QBAN expands and facilities are run in different states we have reached an uncertain time considering who will undertake the administration and management of an Australian Banana clean plant scheme.

On June 30, 2007 the Queensland Banana Industry Protection Board ceased to exist as the Banana Industry Protection Act (1989) was repealed. Since then all of its associated activities and functions, including the QBAN scheme are no longer legally recognised. Due to changes in QBAN administration and in the differences in plant health regulations and procedures in each state it is difficult to collect data on annual Australian banana and tissue culture production. However data for annual plantings in Queensland which is estimated to provide over 80% of Australian production should provide general indications for growth of the Australian industry. Data was collated from department of Primary Industries and Fisheries, Biosecurity Queensland banana plant permit database. The graph below indicates that there has been a steady increase annually in plantings from 1.1 million in 2004 up to a peak of 2.6 million in 2006 which would have been increased due to replant efforts as a consequence of cyclone Larry. Data presented for 2007 is for data up to November (2.2 million plants).



What is significant is the increase in tissue culture percentage of total plant material that is now used by industry. Although data is not complete for 2007, planting material of tissue culture origin accounts for 31 percent of total plantings. This represents a significant increase from past years and in future this figure is likely to remain the same or increase since there appears to be grower recognition of benefits and associated reductions in farm costs especially labour. Although there was slightly lower total percentage of total planting as tissue culture (13%) in 2006 there was an increase in total number of tissue culture plants produced and planted than the previous year. The actual number of tissue cultured plants produced has increased each year since 2004 (refer to graph below). The lower percentage of total therefore was due not to a reduction in tissue culture but to an increase in other types of planting material as industry rushed into a major replant effort. In 2006 the additional demand for plants in the replanting after cyclone Larry meant that there was not sufficient tissue culture supply to meet demand. This was due to the fact that production for tissue culture is planned 12-18 months ahead of supply date and multiplication is restricted to maintain quality and reduce off-types. This combined with some increased losses at culture initiation due to weak suckers after the cyclone meant that tissue cultured plants could not be increased in the short term. However grower orders in 2006 are reflected in the plantings of 2007 where 31% of total industry planting are based on tissue culture.



Discussion and future direction

There has been an increasing demand for commercial banana tissue culture since 2004. The increased upward trend for plants has been exacerbated by cyclone Larry which further increased the need for plants. It will be interesting to see if demand continues to climb. As the demand for tissue culture increases so do the challenges to administer and regulate the QBAN scheme to ensure there is no compromise to plant health quality. Industry need to consider the benefits both from a Biosecurity perspective as well as for improving farm efficiencies that are provided by such a clean plant scheme. The workload to administer and manage

QBAN has and will increase. Demands on the QBAN scheme will also vary over times depending on emerging biosecurity issues which may or may not require increased number of indexing tests to screen for banana plant pathogens. The challenge will be the balance of funding required to meet these additional costs and the need to develop an Australian rather than State based Quality Banana Approved Nursery scheme. With the 2007 planting year relying on 31% of tissue culture for plantings there is now the issue of resources needed to mange this expanding industry sector. Resources provide for this scheme in the past are no longer sufficient to administer and run this successful scheme and there is now an urgent need for industry to resource and move towards development of its own Australian based rather than state based QBAN scheme.

Banana Virology activities

Introduction

Banana suckers intended for tissue culture through QBAN are routinely tested for viruses by the Virology group of QDPI&F (Horticulture and Forestry Science, Indooroopilly). The viruses present in Australia include *Banana bunchy top virus* (BBTV), *Banana mild mosaic virus* (BanMMV), *Banana streak virus* (BSV) and *Cucumber mosaic virus* (CMV). Only BBTV and CMV are regulated as notifiable pests. Of these, BBTV is the most economically important, and nearly wiped out the southeast Queensland/northern New South Wales industries in the 1920s. CMV is widespread in many hosts, but fortunately is relatively uncommon in banana. The other two viruses, BanMMV and BSV, are endemic, but generally occur at a low incidence. In Queensland, field spread of BanMMV (vector unknown) and of BSV by mealybug vectors appears to be negligible. The greatest risk of spread of BanMMV, BSV and CMV is through the use of infected planting material and so quarantine and clean planting material is fundamental to control these viruses.

Bananas coming through post-entry quarantine also require virus indexing, but in this case, all known viruses, including the exotic *Banana bract mosaic virus* (BBrMV), Abaca bunchy top virus and *Abaca mosaic virus* are included. This ensures that the Australian gemplasm collection maintained in this project contains material of the highest health status, and that exotic viruses and new strains of existing viruses are excluded.

Indexing methods

Routine testing of bananas for the QBAN scheme has involved testing by ELISA for BBTV and CMV only. This is because only these two viruses are prescribed pests and represent the most significant threats to industry. The indexing is currently undertaken at below cost. There has been an increase in testing required with approximately 7,600 individual suckers tested over the three years of the project for this scheme. By contrast, post-entry quarantine testing is not subsidized, and targets all known viruses, including general tests to detect possible undescribed viruses.

Financial Year	Suckers collected and sent for virus indexing
04/05	1830
05/06	2950
06/07	2830

PCR tests are now available for all banana viruses and are being implemented for QBAN virus indexing. We have developed a multiplex PCR, which can simultaneously detect BBTV, CMV and BBrMV in the one reaction. A separate PCR has been developed for BanMMV.

BSV is the most difficult banana virus to reliably detect, as it is actually a group of similar viruses (over 20 known at this stage) which can vary dramatically in virus levels in the plant and show very erratic symptom expression. Generic detection of this virus "group" is difficult. ELISA is inefficient as the viruses are serologically variable. Partial purification of the virus and immunosorbent electron microscopic examination is labour-intensive and expensive, but the most reliable method to date. Specific PCR is difficult under these circumstances, but more general group specific PCRs have failed to deliver sufficient test sensitivity. Also, partial genomes of many BSVs are integrated into the banana genome, and in some cultivars, whole genomes are integrated in a latent form, and are able to produce active infections after tissue culture. This phenomenon complicates indexing and the types of assays that can be used, as false positive reactions can easily be obtained. As a compromise, BSV PCR for QBAN currently involves immunocapture and specific assays for the most prevalent viruses.

The QBAN scheme is currently under review, and in the interim, Australian planting material is being indexed by PCR for the four known endemic viruses – BBTV, BanMMV, BSV and CMV. A very low frequency of positive detections of BSV and BanMMV has been noted, after the inclusion of tests for these viruses in routine indexation.

Future directions

The extent of banana virus indexing required for QBAN will be determined in the near future i.e. whether indexing for BSV and BanMMV will be formally included. The detection of BSV and BanMMV in QBAN material, albeit a very low levels, suggests that these tests should be conducted on a routine basis, to enable tissue culture planting material of the highest health status to be produced.

The development of more cost efficient detection assays, such as real-time PCR, is required if virus indexing is to remain both accurate and cost effective.

BANANA TISSUE CULTURE RESEARCH
Improving banana tissue culture production

Evaluating best method for extraction of high quality DNA from banana leaves

Introduction

Subsequent research in this project (chapter to follow) used molecular biology to undertake feasibility studies to investigating improved detection of banana off-types and therefore required adequate amounts of good quality DNA extracted from dwarf off-type and normal banana leaves. Methods of DNA extraction from banana leaves which contain high levels of problematic phenolics were compared using standard methods and commercial kits. Three methods were used to extract DNA from banana leaves these being CTAB buffer extraction method (Graham et al. (1994) DNeasy plant maxi kit (Qiagen) subequently referred to as extraction kit A and Nucleon Phytopure Kit (Amersham Biosciences) subsequently referred to as extraction kit B.

Materials and Methods

The leaf tissue was taken from plants grown t South Johnstone, north Queensland (17°38'S) on a deep alluvial light clay (inceptisol). Plant spacing was 5 m x 1.5 m (1333 plants/ha). The trial area received basal lime applications (2 t/ha), which was broadcast and incorporated prior to mounding and drilling out the rows. An NPK blend (11.1:1.7:17.6) was broadcast in the drill at 500 kg/ha just before planting. Potassium (600 kg/ha/yr as KNO3) plus supplementary nitrogen as urea (total N of 400 kg/ha/yr) was applied in the irrigation water at monthly intervals.

Williams Cultivar (Cavendish AAA subgroup) banana plants from 3 categories were grown in the field at the Department of Primary Industries and Fisheries South Johnstone Research Station. These categories were 1. Normal true-to-type banana plants with no history of production from tissue culture, 2. Dwarf off-types that were produced from TC and 3. Normal true-to-type plants produced from tissue culture. The youngest leaves were collected for DNA extraction and Twelve plants in total were sampled and in some instances samples were taken from both mother and daughter plants (see Table 1). Preliminary samples were taken prior to detailed evaluation from banana plants growing at Maroochy Research Station, Nambour where two types of leaf samples were collected, Maroochy 1 a new unfurled leaf, and Maroochy 2 a new fully open leaf (see Table 1).

Table 1 Banana plant samples for DNA extraction procedures

Sample	Group	Variety
Mother 1	Normal -Never Tissue cultured	Williams
Daughter 1	Normal -Never Tissue cultured	Williams
Mother 2	Normal -Never Tissue cultured	Williams
Doughter 2	Normal -Never Tissue cultured	Williams
Mother 3	Normal -Never Tissue cultured	Williams
Daughter 3	Normal -Never Tissue cultured	Williams
Mother 4	Dwarf off type	Williams
Mother 5	Dwarf off type	Williams
Mother 6	Dwarf off type	Williams
Mother 7	Normal-From tissue culture	Williams
Mother 8	Normal -From tissue culture	Williams
Mother 9	Normal -From tissue culture	Williams
Maroochy1-new unfurled	Normal -From tissue culture	Yangambi KM5
Maroochy2-new opened	Normal -From tissue culture	Yangambi KM5

Youngest leaves were used in all sampling since they produce less obstacles for extraction of DNA and produced the highest yields. Younger leaves also have much less waxy cuticle on the leaf than older leaves which can interfere with extraction of good quality DNA. Young leaves were subsequently used for all further work and collected from the trial block at South Johnstone Research Station. Following collection of the young leaf samples, each leaf was placed into zip lock bags, stored on ice in a polystyrene box and sent overnight by courier to the Maroochy Research Station for DNA extraction.

Initially two DNA extraction techniques were performed in order to determine which technique produces the best quality and quantity of DNA. The first technique is commonly used simple extraction using a detergent, CTAB (cetyl trimethyl-ammonium bromide, Sigma), as the basis for the extraction buffer solution (Graham et al., 1994). The other technique was performed using a commercially available extraction kit A with lysis buffer, salts and filtration steps to allow specific adsorption of DNA and optimal removal of proteins and polysaccharides.

The CTAB extraction method involved using 1g of leaf tissue from each sample ground to a fine powder using liquid nitrogen in a mortar and pestle. The ground leaf powder was quickly transferred to tubes before material thawed and 5ml of CTAB buffer (pH 5.5) added. The CTAB/plant material mix was incubated at 55°C for 20-30 minutes with gentle mixing at least twice followed by centrifuged at 4000 rpm for 20 monutes to pellet cell debris. The clear supernatant from the samples was transferred into clean tubes with the addition of ½ volume of chloroform: isoamyl alcohol (24:1), mixed by inversion to from an emulsion and centrifuged at 400 rpm for 15 minutes. The upper aqueous phase was transferred into clean tubes and the chloroform step repeated once more. The aqueous phase from the final chloroform step was again transferred into clean tubes and the

DNA precipitated with the addition of 1/10 volume of 3M Sodium acetate followed by 2 volumes of absolute ethanol and gentle inversion of the tubes. The DNA was pelleted by centrifuging, washed twice with 70% ethanol, dried using an Eppendorf Speed Vac and finally resuspended in TE buffer (pH 8.0) + RNase.

Following directions from the commercial kit A, the leaf tissue was initially ground using the same technique for the CTAB method. To the ground plant material 5 ml of AP1 buffer and 10μL of RNase A stock solution was added, mixed using a vortex and incubated at 65°C for 10 minutes with the tubes inverted 2-3 times to lyses the plant cells. To the incubated lysate solution 1.8 mL of AP2 buffer was added, incubated on ice for 10 minute and centrifuged at 4000rpm for 15 minutes. The supernatant was decanted into the shredder spin column placed in a tube to collect flow-through, centrifuged at 4000rpm for 15 minutes. The flow-through was transferred to a clean tube with 1.5 volumes of AP3/E buffer added and vortexed immediately. The mix was transferred to a spin column, centrifuged at 4000rpm for 15 minutes the collected flow-trough was discarded and 12ml of AW buffer added to the spin column before centrifuging at 4000rpm for 30 minutes. The flow-through was discarded and the spin column transferred to a clean tube to collect eluates before the addition of 1mL of AE buffer preheated to 65°C and centrifuged at 4000 rpm for 15 minutes. This step is repeated using 0.5mL of AE buffer. The resulting solution contains the purified DNA which was precipitated, washed, dried and resuspended using the same method as performed for the CTAB extraction.

Quantification and purity of the DNA yielded from both techniques was assessed by running 4μL of the DNA solution on a 0.7% agarose gel in 1x TBE and stained with ethiduim bromide (0.5μg/mL).

The concentration of the DNA samples from both extraction methods was determined using a SmartSpec 300 spectrophotometer reading at wave lengths 260, 280 and 320 nm.

A second commercial extraction kit B was also evaluated using the previous methods, in order to ascertain the most suitable means to obtain best quality and quantity of DNA for future analysis. This kit was also described in DNA extraction methods by Peraza-Echeverria et al. (2001). The comparison of CTAB, plant DNA extraction Kits A and B was performed using a single unfurled banana leaf collected from Maroochy Research Station (Table 1), with the leaf cut in half so two extractions were performed on the same leaf sample to ensure results were consistent. The CTAB and Plant DNA extraction Kit A extraction methods were performed as previously described using 1g of leaf tissue. The extraction Kit B method involved adding 4.6ml of Reagent 1 and 1μl of RNase (100mg/ml) to 1g of ground banana leaves (prepared as outlined in CTAB method), mixed by inverting the tube and incubating at 37 °C for 30 minutes. To this mix 1.5mL of Reagent 2 was added, mixed by inverting tubes, incubated at 65°C for 10 minutes before placing tubes on ice for 20minutes. Tubes were removed from ice and 2mL of Chloroform (stored at -20°C) and 200μL of resin was added with regular agitation of tubes during 10 minute incubation at room temperature. The samples were then centrifuged at 2600 rpm for 10 minutes. The upper aqueous phase containing the DNA was transferred into new tubes for DNA precipitation, performed by adding an equal volume of cold isopropanol. The precipitated DNA was removed from solution using a truncated pipette so as not to damage the DNA into an eppendorf tube, pelleted by centrifuge at maximum speed for 5 minutes before being washed, dried and resuspended as performed for the previous methods. Quantification and purity of the DNA yielded from this procedure was again assessed by running on a 0.7% agarose gel as performed for the previous methods.

Results and Discussion

Both the CTAB and extraction kit A methods appeared to produce adequate yields of DNA, however the CTAB method produced higher yields than kit A as evidenced by the thicker banding (Fig 1). The quality of the DNA from the CTAB method also appeared to be of better quality as the DNA extracted from kit A which appeared to be sheared as evidenced by the streaking of the DNA sample down the well on the gel photo (Fig 1&2).

The higher yields produced by CTAB method were confirmed by the concentration readings obtained using the SmartSpec 300 spectrophotometer see Table 2. Analysis revealed that kit A method produced very low yields for all samples ranging from a total of 4.62µg to 31.50µg of DNA in the extracted sample, whereas the CTAB method yields ranged from 175.68µg to 254.98µg.

To recheck the low yields produced by the kit A, the experiment was repeated on all samples, excluding the two Maroochy samples. Again DNA appeared to be sheared (Fig. 3) and the total amount of DNA yielded again was low in comparison to the CTAB method (Table 3).

With the promising results achieved with the CTAB DNA extraction method it was repeated on new samples sent from South Johnstone (Table 1) these extractions were performed in duplicate. The gel (Fig 4 & 5) show this method again yielded good quantities and quality of DNA. When analysed using the spectrophotometer however a more variable range in the total amount of DNA was obtained with readings as low as 12.5µg to 210µg (Table 4) .

The second kit B was subsequently include to compare to other methods. Results of the comparison of the CTAB, kit A and kit B (Fig 6) showed that kit A method again produced sheared DNA but both the CTAB and kit B produced good quality and quantity of DNA.

In future analysis involving DNA digests the CTAB method was deemed the most reliable and time and cost effective method. The CTAB method appeared to be the most effective in delivering DNA of superior quality and quantity compared to that delivered by using the Plant DNA extraction kit A kit and was comparable to the second Plant DNA extraction kit B.

Table 2 Banana DNA extractions results

Sample	Extraction Method	1:10 Conc. (µg/ml	A260/ A280	A320	Stock Conc. (µg/ml)	Total Volume of Sample (µL)	Total Volume of DNA (µg)
Mother 1	KIT A	25.6112	1.939	0.090	256	70	17.92
Daughter 1	KIT A	12.4015	1.961	0.038	124	80	9.92
Mother 2	KIT A	20.7048	2.016	0.088	207	50	10.35
	CTAB	111.7402	1.868	0.139	1117	220	245.74
Daughter 2	KIT A	18.4559	2.003	0.070	184	40	7.36
	CTAB	79.4666	2.203	0.228	794	220	175.68
Mother 3	KIT A	25.6933	1.916	0.105	256	80	20.48
Daughter 3	KIT A	6.6066	2.103	0.072	66	70	4.62
Mother 4	KIT A	16.0536	2.038	0.074	160	60	9.60
	CTAB	115.1880	1.570	0.346	1151	200	230.20
Mother 5	KIT A	13.6975	1.996	0.055	136	150	20.40
Mother 6	KIT A	14.4243	2.035	0.075	144	200	28.80
Mother 7	KIT A	13.8252	2.095	0.069	138	200	27.60
Mother 8	KIT A	15.6226	1.995	0.069	156	80	7.80
	CTAB	112.6253	1.675	0.494	1126	200	225.20
Mother 9	KIT A	21.0314	1.937	0.043	210	150	31.50
Maroochy 1	KIT A	22.0966	1.990	0.102	220	40	8.80
	CTAB	113.3004	1.651	0.304	1133	200	226.60
Maroochy 2	KIT A	28.2354	1.921	0.072	282	40	11.28
	CTAB	115.9465	1.835	0.331	1159	200	254.98

Table 3 Banana DNA KIT A extraction results

Sample	Extraction Method	1:10 Conc. (µg/ml)	A260/ A280	A320	Stock Conc. (µg/ml)	Total Volume of Sample (µL)	Total Volume of DNA (µg)
Mother 1	KIT A	20.0286	1.871	0.024	200	80	16.00
Daughter 1	KIT A	15.1119	1.770	0.024	151	80	12.08
Mother 2	KIT A	24.7741	1.849	0.016	247	100	24.70
Daughter 2	KIT A	14.2486	1.811	0.006	142	80	11.36
Mother 3	KIT A	18.9898	1.822	0.034	189	100	18.90
Daughter 3	KIT A	19.6867	1.855	0.037	196	90	17.64
Mother 4	KIT A	25.5924	1.883	0.052	255	80	20.40
Mother 5	KIT A	38.7261	1.837	0.051	357	80	28.56
Mother 6	KIT A	12.2087	1.830	0.033	122	25	3.05
Mother 7	KIT A	16.9825	1.756	-0.008	169	55	9.30
Mother 8	KIT A	17.6214	1.788	0.012	176	80	14.08
Mother 9	KIT A	16.8810	1.760	-0.003	168	100	16.80

Table 4 Banana DNA CTAB extraction results

Sample	Extraction Method	1:10 Conc. (μg/ml)	A260/ A280	A320	Stock Conc. (μg/ml)	Total Volume of Sample (μL)	Total Volume of DNA (μg)
Mother 1 (M1-1)	CTAB	59.4082	2.081	0.050	594	100	59.40
Mother 1 (M1-2)	CTAB	91.8811	2.059	0.076	918	100	91.80
Daughter 1 (D1-1)	CTAB	31.0246	2.086	0.052	310	100	31.00
Daughter 1 (D1-2)	CTAB	59.1244	2.093	0.078	591	100	59.10
Mother 2 (M2-1)	CTAB	102.5162	1.929	0.073	1025	100	102.50
Mother 2 (M2-2)	CTAB	98.7966	2.046	0.071	987	100	98.70
Daughter 2 (D2-1)	CTAB	12.5850	2.046	0.031	125	100	12.50
Daughter 2 (D2-2)	CTAB	18.8709	3.053	0.040	188	100	18.80
Mother 3 (M3-1)	CTAB	79.3924	2.019	0.094	793	100	79.30
Mother 3 (M3-2)	CTAB	98.9215	2.014	0.144	989	100	98.90
Daughter 3 (D3-1)	CTAB	75.6786	2.119	0.176	756	100	75.60
Daughter 3 (D3-2)	CTAB	56.9886	2.129	0.112	569	100	56.90
Mother 4 (M4-1)	CTAB	66.9392	1.916	0.124	669	100	66.90
Mother 4 (M4-2)	CTAB	109.3697	1.821	0.112	1093	100	109.30
Mother 5 (M5-1)	CTAB	118.7021	1.707	0.117	1187	100	118.70
Mother 5 (M5-2)	CTAB	1:20 - 84.5041	1.947	0.065	1690	100	169.00
Mother6 (M6-1)	CTAB	55.1804	1.962	0.056	551	100	55.10
Mother6 (M6-2)	CTAB	71.1406	1.961	0.083	711	100	71.10
Mother 7 (M7-1)	CTAB	81.7775	1.974	0.119	817	100	81.70
Mother 7 (M7-2)	CTAB	64.0846	1.959	0.110	640	100	64.00
Mother 8 (M8-1)	CTAB	1:20 - 81.2576	1.929	0.064	1625	100	162.50
Mother 8 (M8-2)	CTAB	1:20 - 105.1433	1.863	0.112	2102	100	210.20
Mother 9 (M9-1)	CTAB	1:20 - 64.6853	1.948	0.044	1293	100	129.30
Mother 9 (M9-2)	CTAB	1:20 - 99.6485	1.910	0.110	1992	100	199.20

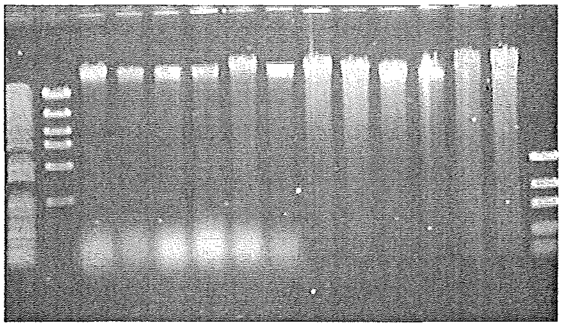


Figure 1 – CTAB and PLANT DNA EXTRACTION KIT A DNA extraction quantification and quality assessment. Lane 1: molecular weight marker 1 kb Plus (Gibco BRL), Lane 2: high mass ladder, CTAB - Lane 3: Mother 1, Lane 4: Daughter 2, Lane 5: Mother 4, Lane 6: Mother 8, Lane 7: Maroochy 1, Lane 8: Maroochy 2, PLANT DNA EXTRACTION KIT A Lane 9 Mother 2:, Lane 10: Daughter 2, Lane 11: Mother 4, Lane 12: Mother 8, Lane 13: Maroochy 1, Lane 14: Maroochy 2, Lane 15: low mass ladder

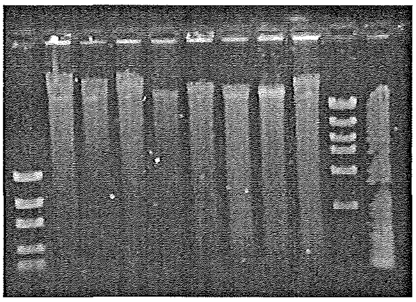


Figure 2 - PLANT DNA EXTRACTION KIT A DNA extraction quantification and quality assessment Lane 1: molecular weight marker 1 kb Plus (Gibco BRL), Lane 2: Mother 1, Lane 3: Daughter 1, Lane 4: Mother 3, Lane 5: Daughter 3, Lane 6: Mother 5, Lane 7: Mother 6, Lane 8: Mother 7, Lane 9: Mother 9, Lane 10: high mass ladder, Lane 11: low mass ladder.

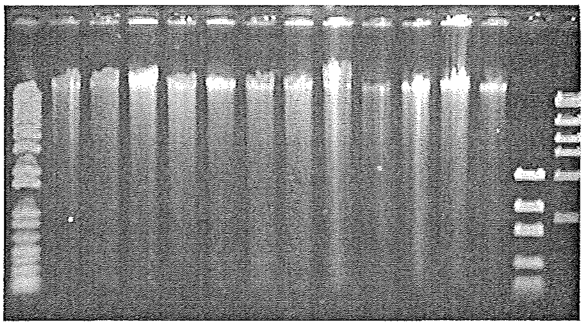


Figure 3- PLANT DNA EXTRACTION KIT A DNA extraction quantification and quality assessment Lane 1: molecular weight marker 1 kb Plus (Gibco BRL), Lane 2: Mother 1, Lane 3: Daughter 1, Lane 4: Mother 2, Lane 5: Daughter 2, Lane 6: Mother 3, Lane 7: Daughter 3, Lane 8: Mother 4, Lane 9: Mother 5, Lane 10: Mother 6, Lane 11: Mother 7, Lane 12: Mother 8, Lane 13: Mother 9, Lane 14: low mass ladder, Lane 15: high mass ladder,

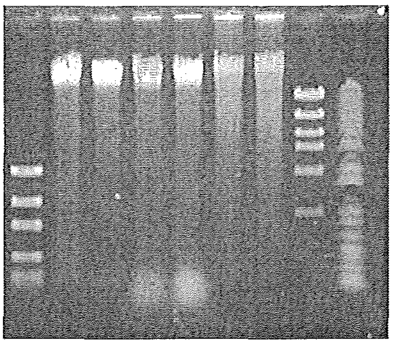


Figure 4 – CTAB DNA extraction quantification and quality assessment. Lane 1: low mass ladder, Lane 2: Mother 1, Lane 3: Mother 1, Lane 4: Mother 2, Lane 5: Mother 2, Lane 6: Mother 3, Lane 7: Mother 3, Lane 8: Daughter 1, Lane 9: Daughter 1 , Lane 10: Daughter 2, Lane 11: Daughter 2, Lane 12: Daughter 3, Lane 13: Daughter 3, Lane 14: high mass ladder, Lane 15: molecular weight marker 1 kb Plus (Gibco BRL).

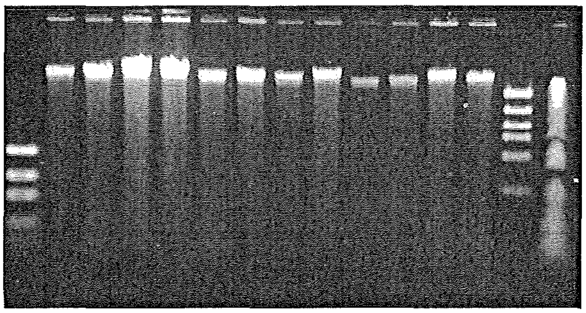


Figure 5 – CTAB DNA extraction quantification and quality assessment. Lane 1: low mass ladder, Lane 2: Mother 4, Lane 3: Mother 4, Lane 4: Mother 5, Lane 5: Mother 5, Lane 6: Mother 6, Lane 7: Mother 6, Lane 8: Mother 7, Lane 9: Mother 7, Lane 10: Mother 8, Lane 11: Mother 8, Lane 12: Mother 9, Lane 13: Mother 9, Lane 14: high mass ladder, Lane 15: molecular weight marker 1 kb Plus (Gibco BRL).

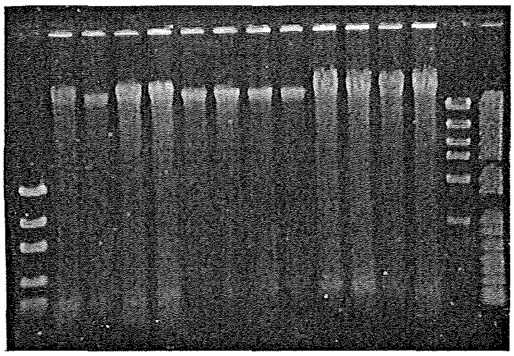


Figure 6 – CTAB, PLANT DNA EXTRACTION KIT A and PLANT DNA EXTRACTION KIT B extraction quantification and quality assessment. Lane 1: low mass ladder, Lane 2: Kit B, Lane 3: Kit B, Lane 4: C1, Lane 5: C2, Lane 6: Kit A, Lane 7: Kit A, Lane 8: high mass ladder, Lane M: molecular weight marker 1 kb Plus (Gibco BRL).

Evaluation of molecular methods for better understanding somaclonal variation in banana tissue culture

Introduction

Somaclonal variation (SV) is a significant obstacle to wide spread uptake of tissue culture planting material. The molecular basis of SV is not precisely known, but appears to involve both genetic and epigenetic mechanisms. Labile portions of the genome apparently can be modulated when cells undergo tissue culture stress. Therefore, predictable DNA markers should be able to differentiate between 'normal' and 'off-type' tissue cultured plants. Ongoing research into banana tissue culture production and quality issues including molecular biology approaches to better understand SV aims to overcome impediments to grower use.

1. Conversion of a negative to a positive PCR marker for off-types

For banana tissue culture to be more readily adopted, some quality issues need to be overcome. We have identified a need to understand when banana off-types occur in the tissue culture process. In previous work, we have identified a molecular marker for dwarf off-types that is manifested by the <u>absence</u> of a particular marker in the dwarf (Damasco et al., 1996, Damasco et al., 1998 and Ramage et al., 2004) but it is expensive to use since it requires screening of individual plantlets. To be able to screen batches of plants at one time a positive marker will be required. A 'positive' marker for the dwarf off-type of Williams would contribute to management of off-types to increase percentage of true-to-type plants reaching the grower and to assist in identifying steps during tissue culture that lead to production of dwarf off-types.

Materials and Methods

A method for the conversion of negative (missing marker) to positive DNA markers in banana (Umali et al., 2002) was published in the scientific literature. We have attempted the described approach to develop and validate a positive DNA marker for dwarf off-types from the commercial Williams variety. To achieve this, the 1625 nucleotide sequence of the negative marker (Ramage et al. 2004) has been used to design new, overlapping primers using Primer3 software online. Genomic DNA from replicated known dwarf off-types and their normal parents has been extracted as described in the previous sections and was used as template in polymerase chain reaction for marker identification and verification.

Polymerase chain reaction assays were done in a Perkin Elmer model 9700 thermal cycler using the following conditions: 94°C for 2 min, 40 cycles of 94°C for 40 sec, 55°C for 30 sec and 72°C for 1 min.

PCR mix (25μl) contained: 2.5μl 10X buffer
1μl 5 mM dNTP (200μM final)
10μl 5uM primer (2μM final)

1.5μl of 1:10 dilution of DNA sample
0.3μl Taq DNA polymerase (1.5 units)
9.7μl deionised water

Results

1. Single primer PCR was used to generate a unique band(s) specific for the dwarfing phenotype. For the initial experiments, primers B915, B1035, B920 and B509 (Table 1, panel A) were used with DNeasy extracted DNA from M1, 2 and 3 (from normal plants) and M4,5 and 6 (from dwarf off types). Only results with primer B1035 were encouraging as they showed a doublet of PCR products about 1Kb in size only in the three dwarf off-type DNA samples.

2. To further optimise PCR conditions, a range of annealing temperatures from 50-60 degrees were tested with the same conditions and using additional DNA samples with primer B1035. Annealing temperatures were 50, 53, 57 and 60 degrees in a temperature gradient cycler (Corbett Research). Results from this experiment did not show any bands that were exclusively present in dwarf off–type samples.

3. To validate the previous results, CTAB extracted DNA samples were used instead of DNeasy extracted DNA. Primer B1035 primer was used with the same conditions as in experiment 1 and an annealing temperature of 50 degrees. Result was same as in experiment 2, with no clearly distinct bands exclusively in dwarf off-types.

4. Five new overlapping primers, L571, L705, L874 and L918 were designed (Table 1, panel B) and tested in single primer PCRs using the same conditions as previously using CTAB-extracted DNA samples. No bands exclusive to DNA from dwarf off-types were identified.

5. Four new primers, L293, R724, R934 and L922 were designed (Table 1, panel; C) and tested in single primer PCRs using same conditions as previously but using DNeasy extracted DNA samples. However, once again no distinct bands unique to off-type samples were observed.

Table 1. Oligonucleotide primers used in this study.

	Panel A
B915:	5'- CTC CAT TCT TGC CCT CAC TC-3'
B1035:	5'- GTT GTT GCT CGC TCC ATT CT-3'
B920:	5'- TTC TTG CCC TCA CTC GTC TC-3'
B509:	5'- GCC TTG GAT TGA CTG CTT TC-3'
	Panel B
L918:	5'-CAT TCT TGC CCT CAC TCG TC-3'
705:	5'- ACA TAG CAG CAG TCC CAT CC-3'
L571:	5'- AGT CTG ATG GTA GTG GTT GC-3'
L874:	5'- CTG GTT CTG TAA CTC CCT TG-3'
	Panel C

L922:	5'-CTT GCC CTC ACT CGT CTC A-3'
L293:	5'- AGG CTT AGT GGC TGT AGT TG-3'
R724:	5'- GGA TGG GAC TGC TGC TAT GT-3'
R934:	5'- GAG TGA GGG CAA GAA TGG AG-3'
	Panel D
293.R:	5'- GGC TAT CCG TTG CTT GTT G-3'
293-F2:	5'- GGC TTA GTG GCT GTA GTT-3'
293-R2:	5'- CTT GCC TAT CCG TTG CTT-3'
293.Flg:	5'- AGG CTT AGT GGC TGT AGT TGA AGC-3'
934.Rlg:	5'- GAG TGA GGG CAA GAA TGG AGG CAA GGG CAG-3'

6. Two primer PCRs were done using pair wise combinations of primers previously designed. DNeasy extracts from two normal plants and two dwarf off-types and the previous cycling conditions were used. Primer R934 was used in combination with either L293, L871, L571, L705, L509 or L1035. The primer pair R934 & L293 yielded a faint unique band in the two off-type samples which did not appear to be present in the two normal plant samples.

7. Repeat experiments with this primer combination (R934 & L293) and with three DNA samples each from normal and off-types revealed a unique band consistently in all off-type samples. The DNA band was gel isolated and cloned, but no recombinant clones were obtained due to technical difficulties.

8. Additional PCRs were done using primer R724 in combination with either L1035, L509, L915, L920, L874, L293,L574, L922 or L918. Primer pairs R724 & L509 and R724 & L293 yielded faint apparently unique bands with the off-type samples.

9. PCR assays were repeated and again primer combinations R724 & L509 and R934 & L293 showed clear unique bands in the off-type samples. The bands were gel isolated and the PCR product of R934 & L293 was successfully cloned and sequenced.

10. The DNA fragment amplified from dwarf off-type DNA using primers R934 & L293 was 1554 bp in size; it had no sequence similarity to the original 'negative' marker sequence. This "dwarf off type-specific" sequence was used to design three new primers, 293F2, 293R and 293.R2 (Table 1, panel D) to develop smaller, specific PCR markers.

11. Using these and additional new primers five primer combinations were tested in PCR:

293.F2 & 293.R2
293.F & 293.R2
293.F2 & 293.R
293.F & 934.R
293.F2 & 934.R

The best combinations that yielded unique bands in dwarf off-types were 293.F & 934.R and 293.F2 & 934.R.

12. To improve band intensity for clearer results, a temperature gradient from 60-70 degrees annealing was tested (60, 63, 65, 68, 70°C) using same conditions as before. 65°C annealing temperature yielded the strongest band.

13. The dwarf off-type specific bands were gel isolated, cloned and sequenced. Using this sequence, slightly longer primers were designed for use at higher annealing temperatures –

293.Flg and 934.Rlg. Use of these primers with DNeasy extracted DNA at 60°C annealing and with 30 PCR cycles, however, did not show any clearly distinct bands in dwarf off-type samples.

Discussion

Use of a previously identified molecular marker sequence for dwarf off-types that is manifested by the absence of a particular band in the dwarf as the basis for development of a 'positive' marker that is present in dwarf off-types and absent in normal bananas, was unsuccessful. The single overlapping primers we designed did not yield any dwarf-specific DNA bands (experiments 1-5). We therefore changed our approach to use primer pairs designed from the same initial sequence. Three primer pairs resulted in apparently unique 'dwarf off-type-specific' bands; these bands from two primer pairs were isolated, cloned into plasmids and their nucleotide sequence was determined. However, primer pairs designed from these putatively unique sequences to yield smaller PCR products which could be used as molecular markers in routine, high throughput testing, did not yield clear and reproducible results.

2. Analysis of global methylation polymorphisms

We also aimed to determine if off-types have changed genome methylation patterns which can be identified using Amplified Methylation Polymorphism (AMP) markers, ie. find out if there is a correlation between dwarf off-type markers and AMP profiles. We hypothesized that there is a link between tissue culture, somaclonal variation and methylation changes in banana. This technique would then be used to identify the cause(s) and incidence of methylation changes which in turn could be used to revise particular tissue culture steps to improve the quality of micropropagated bananas.

Preliminary experiments using fluorescently labelled primers indicated a degree of polymorphisms, but the DNA quality was not sufficient to allow unambiguous identification of unique polymorphisms distinguishing DNA derived from non-tissue cultured, dwarf off-types and plants grown from tissue cultured parents. An improved DNA purification method was provided in confidence from a colleague and new samples were extracted. However, due to unexpected staff changes, we were unable to continue these experiments.

Bacterial indexing of banana growing points used to establish tissue cultures

Introduction

Bacterial contamination is a major problem associated with plant tissue culture, decreasing explant multiplication rates or leading to loss of plantlets. Strategies to control bacterial contamination increase production costs in both commercial production and research (Van den houwe et al., 1998; Trick and Lingens, 1985). Bacterial contamination may be introduced to the culture through the explant tissue itself as rhizosphere microbes colonising the surface of the plants in their natural environment, as endophytic bacteria residing within the plant tissue, or from the laboratory environment during tissue preparations. Contaminants may either grow immediately, making control relatively easy, or in many cases remain latent for long periods of time making the identification and management of contamination extremely difficult (Cassells 2001; Leifert et al., 1994). Because contamination provides a significant impact on production reliability and costs of production a reliable indexing is required. Previous studies were conducted into the characterisation and control of bacterial contaminants from *in vitro* cultures of banana stored in a germplasm collection by Van den houwe et al., (2000), as well as investigation of quality assurance systems for plant cell and tissue cultures (Leifert and Woodward, 1998). In these studies Gram-positive endospore-forming *Bacillus* sp. (65%) and the slow-growing *Mycobacterium* sp. (30%) predominated, comprising as much as 95% of the contaminants identified. Various methods to eliminate bacteria have been investigated. Antibiotics have been found to be largely unsuccessful in the elimination of bacterial pathogens from established plant tissue cultures, (Gilbert et al., 1991) and in many cases only inhibit bacterial growth resulting in persistent low levels of contamination. It has been found that using meristems of approximately 1mm was effective in eliminating 82-100% of *Bacillus* sp. but only 0-55% of *Mycobacterium* spp. (Leifert 2000; Van den houwe and Swennen, 2000). The subculturing of banana meristems smaller than 1mm in size was found to be impractical as survival rates of plant tissue at this stage only averaged 72-85% (Van den houwe and Swennen, 2000).

Current bacterial reduction strategies based on meristem tissue culture can reduce contamination in banana tissue cultures, but fail to eliminate latent bacteria and sporadic, seemingly random contamination still occurs. Such contamination is a major source of concern for laboratories maintaining germplasm in long term *in vitro* storage and for research requiring material that is free from bacteria (such as cryopreservation or somatic embryogenesis). The fact that in most cases bacterial contamination remains undetectable, both visually and using cultural isolation methods, until accessions have been in culture for several months, means that large numbers of propagules may have to be discarded after significant resources have been used. Undetected contamination of important varieties stored in germplasm banks may result in their loss, and also runs the risk of latent pathogenic bacteria being unwittingly transported across quarantine zones (Cooke et al. 1992). Ideally the bacterial status of the banana accessions should be established when they are first initiated into culture or enter the laboratory.

The question remains as to whether cultures that appear uncontaminated are actually free from bacteria, or retain low levels of bacteria that later become problematic when cultures are either stressed or conditions become more amenable for bacterial growth? A screening technique to detect bacteria in the initial explant and source material (suckers) would be a useful tool in banana research. A more effective method of detecting bacteria would allow work to progress in the understanding of sources and mechanisms of bacterial infection and subsequent triggers to the proliferation of bacterial contaminants. Current culture dependent screening methods such as streaking explants onto bacterial growth media have not been successful in detecting the latent bacteria that are observed in culture many months later. An improved method to either eliminate bacteria at culture establishment, or to detect bacteria remaining at initiation, is needed. Previous work by Hamill et al. (2004, 2005), has identified methods for improved isolation of bacteria. Further research has been undertaken to establish the effectiveness of the indexing method considering future commercial application. This work investigates methods to identify bacteria at culture initiation rather than after culture establishment and to understand levels and types of bacteria living in banana suckers.

Materials and methods

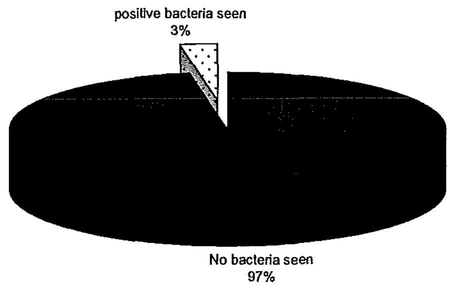
1444 banana suckers were collected for tissue culture initiation and bacterial indexing from 878 suckers from a diverse range of cultivars came from a banana field collection, South Johnstone, North Queensland (17°S, 145°E) and 570 suckers from Williams (Cavendish AAA) were sourced from commercial grower properties located in Tully for an experiment comparing different initiation methods (meristem verses shoot-tip). All cultures were intitated using meristem culture apart from 252 suckers that were initiated as shoot-tip cultures. Generally 5 suckers were selected for each accession to be screened from individual mother plants. All suckers were similar in size (ave. 250mm x 90mm) and cleaned to remove excess soil and tested negative for Banana Bunchy Top Virus, Cucumber Mosaic Virus, Banana Mild Mosaic Virus and Banana Streak Virus. Under aseptic conditions a tissue block containing the meristem was taken from each sucker, surface sterilised with 2.5% v/v sodium hypochlorite for 15 minutes, rinsed 3 times with sterile distilled water prior to excision of the meristem (diameter approx. 1mm) or the block of tissue containing the meristem for shoot-tip culture (25 mm x 10 mm). The banana plantlets were cultured as described by Hamill *et al.* (1992). Cultures were maintained on Murashige and Skoog medium (1962) containing 2.5 mg/l Benzyl amino purine and 1 mg/l Indoleacetic acid with 20g/l sucrose.

Bacteria were isolated from the freshly excised meristems or shoot-tip cultures by streaking the base of the explant onto nutrient broth agar (beef extract, 3 g l^{-1}; peptone 5 g l^{-1}; sodium chloride, 8 g l^{-1}; yeast extract 2 g l^{-1}; with or without bacteriological agar, 15 g l^{-1}). and two tissue sections of approximately 1 mm^2 were extracted aseptically at the time of culture initiation adjacent to the meristem in meristem culture or mid section of the block of tissue for shoot-tip culture, visual bacteria contamination was also monitored in the newly initiated cultures as well as subsequent to culture establishment. Tissue sections were macerated with a sterile scalpel and inoculated to either nutrient broth or nutrient broth solidified with. Meristems (1mm) were cultured on Murashige and Skoog (1962) culture media. All cultures were incubated under tissue culture conditions of 28 ^{o}C, with nutrient broths shaken at 100 rpm.

Results

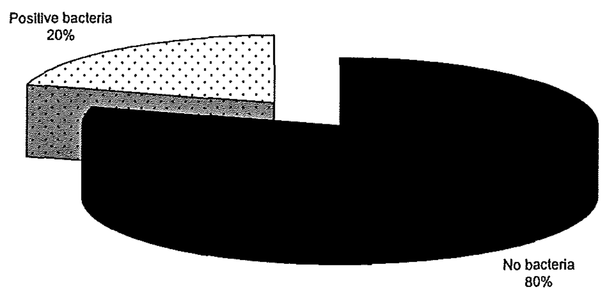
Meristem culture while reducing bacterial contamination does not eliminate it. Visual observation alone, where cloudiness or bacterial colonies were observed, in screening of 1444 cultures in the first 6 weeks after culture initiation (three x 2 weekly subculture) detected 3% of bacteria in cultures.

Number of suckers where bacteria observed in 1444 suckers screened using visual inspection only



However these 1444 suckers were also screened for bacteria using three additional indexing methods: streaking of explant base, maceration of tissue adjacent to meristem into nutrient agar and into nutrient broth. By using all four indexing methods the number of suckers identified with bacteria was increased to 20%.

Number of suckers where bacteria was observed in 1444 suckers screened using all indexing methods



The graphs above indicate number of suckers with bacteria but not number of bacteria that were isolated. All combinations of indexing methods were useful in isolating bacteria to varying degrees. In other words sometimes individual methods isolated bacteria or different combinations of indexing methods were effective and occasionally all of the 4 methods on an individual sucker cultured bacteria. Of the 1444 suckers indexed, 525 bacteria were isolated from various combinations of indexing methods from 296 suckers that were then removed from production.

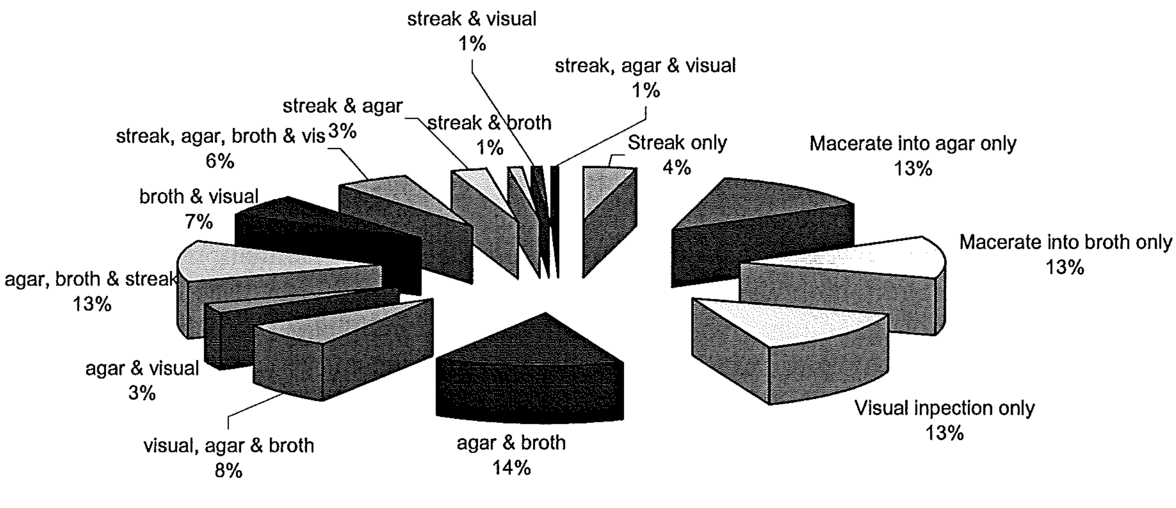
Approximately 416 suckers initiated between 1999-2003 have been maintained as 104 accessions, where suckers from the same mother plant were bulked together. These suckers were initated by meristem culture but were subject only to visual indexing method. Visual contamination has been observed in 3.8% of these accessions.

Of the suckers that were screened using all 4 methods of indexing and where no bacteria was found, approximately 632 suckers were maintained in culture as 158 accessions such that suckers from the same mother plant were bulked together. These banana cultures have been maintained starting in 2004 and to date there have been no cultures showing visual indications of bacterial contamination.

Indexing method	**Number of suckers where found**
Streak	79
Macerate tissue into agar	157
Macerate tissue into broth	177
Visual observation	112
Total number suckers	296
Total number bacteria	525

The trends of effectiveness of the various combinations of indexing methods is tabulated below and shown graphically.

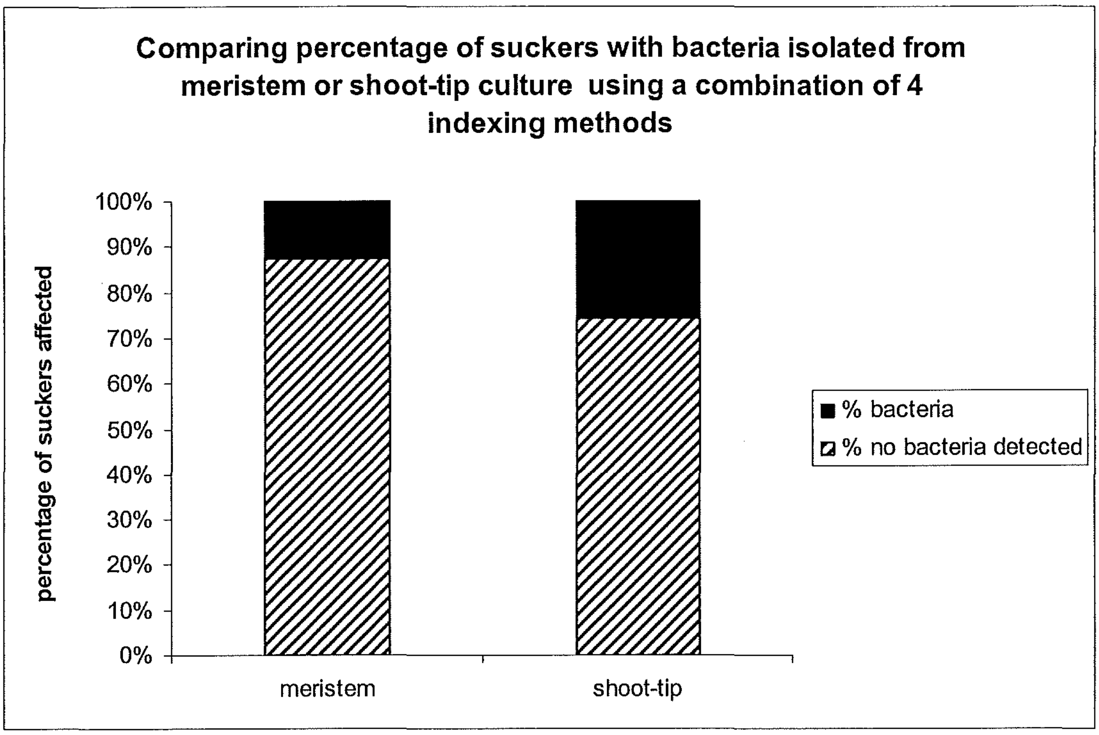
Percentage of suckers where bacteria was isolated from combinations of indexing methods in 256 out of 1444 banana suckers.



Combinations of indexing methods where bacteria was found in each method	Number of suckers
Streak only	12
Macerate into agar only	39
Macerate into broth only	38
Visual inspection only	41
agar & broth	44
visual, agar & broth	23
agar & visual	9
agar, broth & streak	37
broth & visual	20
streak, agar, broth & visual	17
streak & agar	9
streak & broth	3
streak & visual	2
streak, agar & visual	2
Total number of suckers	296

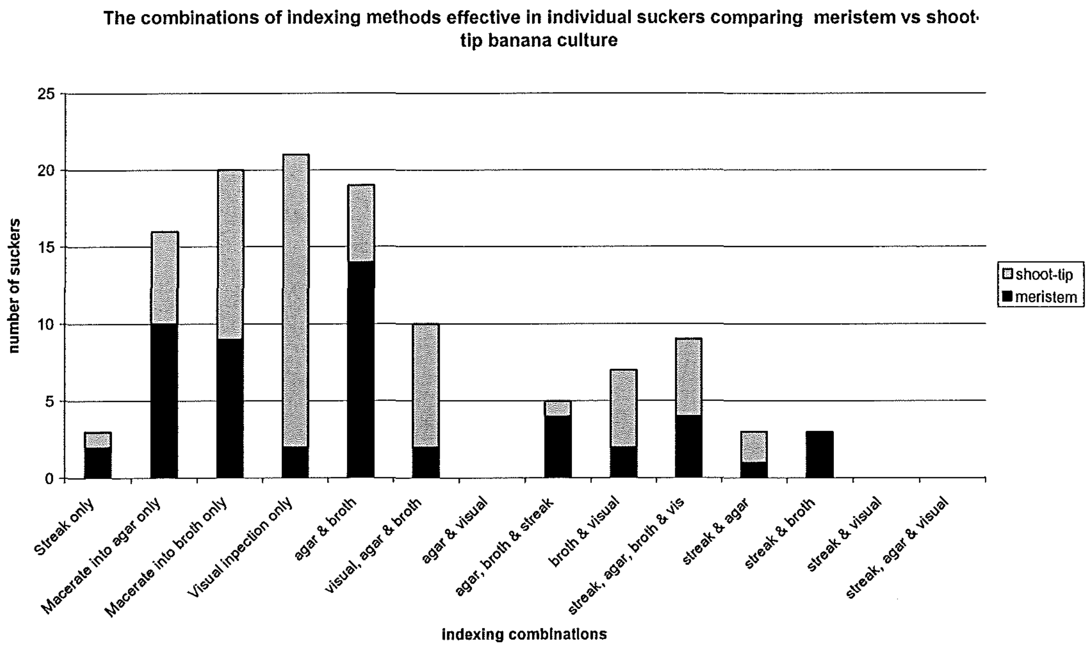
While meristem culture is used routinely to reduce bacteria in our Australian germplasm collection this is not the preferred method of commercial initiation. Since reliability of commercial production of plants is a major issue facing industry it is important to develop an indexing protocol that also has practical application in the commercial sector. Therefore an experiment was conducted comparing indexing methods on meristem verses shoot-tip culture. 4 indexing methods were applied to each of 309 suckers used for meristem culture and 257 suckers used for shoot-tip culture.

13 % of suckers initiated by meristem culture indexed positive to bacteria. 25% of suckers initiated by shoot-tip culture indexed positive to bacteria. However meristem culture requires a higher level of skill in culture initiation and 2.6% of suckers were damaged using meristem culture compared to 1.9% in the shoot-tip culture treatment. Overall loss due to combined bacteria contamination or damage during initiation was 15.6% loss using meristem culture verses 26.9% loss using shoot-tip culture. This resulted in an increase in clean established cultures obtained by meristem culture compared to shoot-tip.

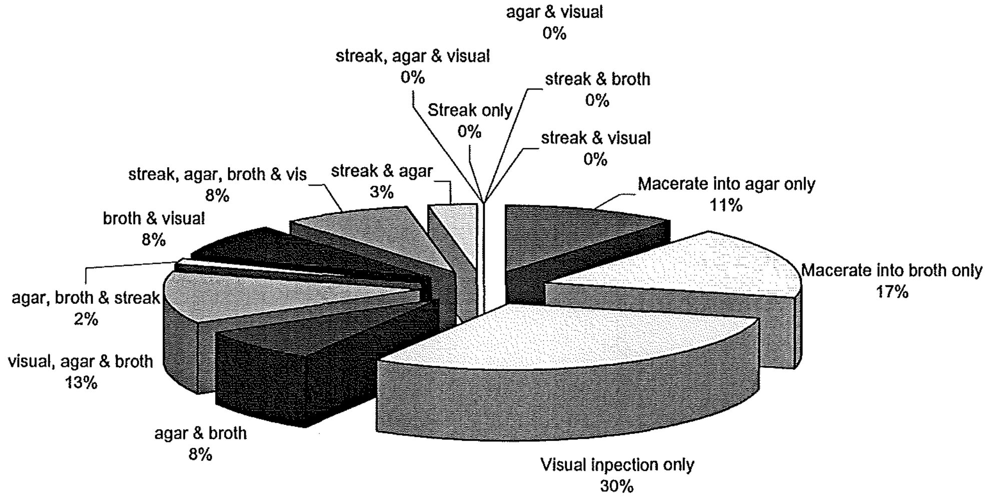


The effectiveness of individual indexing methods varied significantly comparing meristem to shoot-tip culture The graph below indicates numbers of suckers for each treatment were only one of the four indexing methods applied was effective in isolating bacteria.

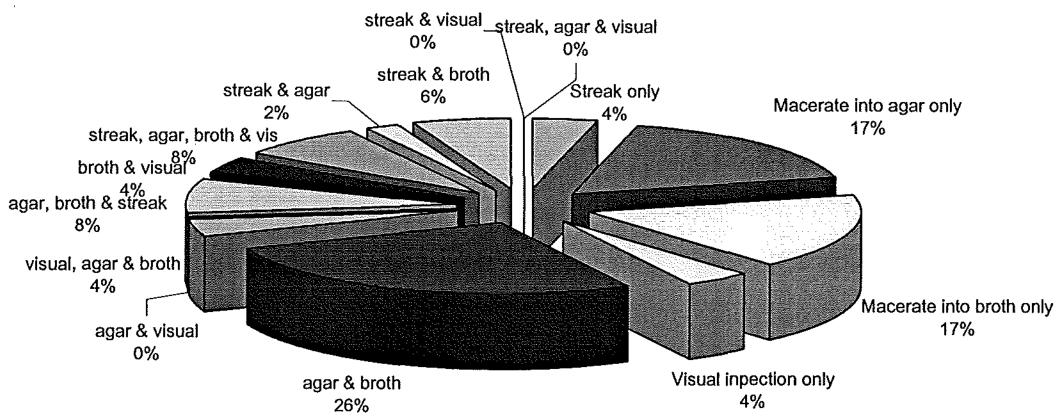
However many suckers had bacteria isolated from more than one method at the time of screening. The number of suckers where various combinations combinations of indexing methods were effective is described in the graphs below.



Percentage of banana suckers used to initiate shoot-tip cultures where combinations of indexing methods isolated bacteria



Percentage of individual banana suckers used for meristem culture where combinations of indexing methods isolated bacteria



Discussion and conclusion

Bacterial indexing when all four methods are applied provides significant improvements in the detection of bacteria in banana suckers that are used for tissue culture initiation. 3% of suckers were identified with bacteria using visual inspection on meristem cultures initiated from banana suckers but when three additional methods (streaking tissue adjacent to mersitem, macerating tissue adjacent to meristem into agar or broth) were also used up to 20% of suckers were identified with bacteria. This represents a substantial improvement with great potential to reduce contamination losses during the tissue culture production cycle. For improved efficiency it would be better if a lower number of screening methods could be used to detect bacteria, however all 4 methods are needed to detect bacteria. The macerated tissue in either nutrient broth or nutrient agar isolated the majority number of bacteria, using these two methods only, accounted for the majority ((92%) of bacteria found. Visual observation combined with these two methods accounted for a further 4% of bacteria isolated and streaking as the only method accounted again for an extra 4% of bacteria isolated from suckers. Traditional methods of streaking and observation in tissue adjacent to the meristem in the banana suckers used for culture initiation accounted for only 8% of the total bacteria isolated and are not reliable methods for identification of bacteria.

Three years after tissue culture initiation cultures from suckers that indexed negative to bacteria using all four indexing methods have remained visually free from contamination. It is important therefore to use all indexing methods to detect all bacteria in banana suckers since all combinations of methods were successful in isolating bacteria. Detection of bacteria in the initial starting material used for plant production will allow laboratories to remove contamination and avoid losses by bacteria that typically occur towards the end of the production cycle. By reducing losses due to bacteria commercial laboratories can become more cost efficient as well as provide a more reliable supply.

Considering the importance of commercial production it is therefore important that indexing methods are applicable to commercial laboratory practices. Generally commercial laboratories initiate banana cultures by shoot-tip culture rather than meristem culture so indexing methods must be aimed at these practices. Comparing meristem to shoot-tip culture indexing methods showed a difference in effectiveness dependant on the size of explant used for culture initiation. In shoot-tip culture 30% of bacteria were identified using visual observation alone compared to visual alone detecting only 4% of the bacteria in meristem culture. Therefore indexing methods detected a further 70% of the bacteria in shoot-tips compared to a massive 96% bacteria in meristem culture. Another issue is that more suckers were discarded due to bacterial for shoot-tip culure (% 26) with only half this amount (% 13) having to be discarded with meristem culture.

In both cases it is obvious that bacterial indexing can be used to dramatically reduce the number of infected suckers at initiation of banana tissue cultures. Meristem culture is the more effective way of reducing the contamination and therefore retaining more suckers that can be used for culture production. Further work needs to evaluate how each of these methods perform under commercial production situations considering multiplication and overall loses due to bacteria. Consideration of commercial issues will allow us to develop the best and most practical method for application to not only research but for commercial improvements in banana tissue culture production.

Optimisation of 16S rDNA PCR and sequencing of bacteria

Introduction

The gene that encodes the bacterial small ribosomal RNA subunit (16S rDNA) has been sequenced for many bacteria. The sequence of this gene is highly conserved among closely related bacterial species. As such 16S rDNA sequencing is used extensively for phylogenetic analysis and identification of bacteria.

The objective of this work was to optimise the use of 16S rDNA sequencing to identify bacteria isolated from banana meristem and shoot tip cultures. A commercial kit was used for the isolation and purification of high quality genomic DNA for optimisation of 16S rDNA PCR and sequencing procedures. Primers 27F and 1492R were used to amplify almost the entire 16S rRNA gene by PCR (Girfoni *et.al.* 1995; Weisburg *et.al.* 1991). A further primer, 926R (Muyzer *et.al.* 1993) was used for direct sequencing of the purified PCR product.

Materials and Methods

Bacterial Cultures

Bacterial cultures were streaked onto nutrient agar (beef extract, 3 g l^{-1}; peptone 5 g l^{-1}; sodium chloride, 8 g l^{-1}; yeast extract 2 g l^{-1}; with bacteriological agar, 15 g $l^{-1)}$ to obtain single colonies and incubated at 28°C overnight for faster growing bacteria or up to seven days for slower growing bacteria. Streaking was repeated three times or until the culture was pure.

DNA Extraction

Bacterial genomic DNA was extracted from both colonies growing on nutrient agar and from cells of an overnight culture, using the GenElute Bacterial Genomic DNA Kit according to manufacture's instructions as follows. Pipette tips with an aerosol barrier were used throughout the procedure. Cells were harvested from 1.5ml of overnight culture by centrifuging at 14 000 X *g* for 2 minutes. The supernatant was discarded and the pellet was resuspended in 200 µL of freshly prepared Lysozyme solution (2.115 X 10^6 units/ml). Alternatively a bacterial colony was resuspended in 200 µL of freshly prepared Lysozyme solution. This suspension was incubated at 37°C for 30 minutes. 20 µL of RNase A solution was then added and the suspension was incubated at room temperature for 2 minutes. Proteinase K (20 µL) and Lysis solution C (200 µL) were added. After thorough pipette mixing the suspension was incubated at 55°C for 10 minutes.

During this incubation 500 µL of Column Preparation Solution was added to each GenElute Miniprep binding column seated in a 2 ml collection tube. The column was centrifuged at 14 000 X *g* for 1 minute and the eluate was discarded. 200 µL of ethanol (100%) was added to the lysate and the suspension was mixed thoroughly for 10 seconds before it was loaded onto the binding column. The column was centrifuged at 6500 X *g* for 1 minute. The collection tube containing the eluate was discarded and the column was placed in a fresh collection tube.

Wash solution 1 (500 μL) was added to the column. The column was centrifuged at 14 000 X *g* for 1 minute. The collection tube containing the eluate was discarded and the column was placed in a fresh collection tube. The second wash was performed by adding 500 μL of Wash solution 2 (containing ethanol) to the column and centrifuging at 14 000 X *g* for 1 minute.

The column was placed in another fresh collection tube. Elution solution (200 μL) was added to the column, incubated at room temperature for 5 minutes and centrifuged for 1 minute (6500 X *g*). The eluate contained the purified gDNA. An aliquot of this DNA was stored at 2-8 °C for short term use. A further aliquot was kept at -20 °C for long-term storage.

Agarose Gel Electrophoresis
The gDNA was visualised by agarose gel electrophoresis with ethidium bromide staining. 5 μL of the neat DNA and 5 μL of a 1:10 dilution of the DNA were run on a 1% (wt/vol) agarose gel prepared with 1 X TAE and amended with 0.5 ug/ml of ethidium bromide. The gel was run with a Lambda DNA/ EcoRI-*Hind*III marker or High mass ladder (MBI) in 1X TAE running buffer at 100V for 45 minutes.

16S rDNA PCR
The 50μL 16S rDNA PCR reaction was comprised of 1X PCR Supermix (20mM Tris-HCl, 5mM KCL, 1.5mM, 200μM of each dNTP, 1 U Taq DNA polymerase; Invitrogen, California), 1μM of each primer :
27F'AGAGTTTGATCCTGGCTCAG-3'; Sigma-Genosys, Australia
1492R 'CGGCTACCTTGTTACGACTT-3'; Sigma-Genosys, Australia,
3.2μL of template DNA (diluted 1:10) and an additional 0.5mM $MgCl_2$ to produce a final concentration of 2mM $MgCl_2$. Pipette tips with an aerosol barrier were used to set up the PCR reaction. In the control reaction DNA was replaced with sterile MQ water. Cycling conditions included an initial denaturation step at 94 °C for 4 min; 29 cycles of 94 °C for 45sec, 60 °C for 45 sec, 72 °C for 90sec; and a final extension at 72 °C for 10min. Cycling was performed in a PC 960G thermal cycler (Corbett Research, Australia). The PCR products were visualised on a 1% agarose gel that was stained with ethidium bromide as described earlier.

Sequencing of PCR products
The PCR products were purified and then sequenced with an ABI 3730XL using ABI PRISM Big Dye Terminator Sequencing Chemistry (Version 3.1) by Macrogen Inc (Korea). The primer 926R (5'-CCGTCAATTCCTTTGAGTTT-3') was used for sequencing.

Sequence Analysis
Sequence chromatograms were inspected using the program Finch TV (www.geospiza.com/finchtv/index.htm). The Basic Local Alignment Search Tool (BLASTn) at the National Centre for Biotechnology Information (http://www.ncbi.nml.nih.gov/BLAST) was used to compare sequences (300-500bp) to those in GenBank. The distance tree produced by BLAST was used to

infer the phylogenetic relationship of isolates with nearest neighbours (most closely related species). Sequences were also analysed with the SeqMatch and Classifier programs at the Ribosomal Database Project (http://rdp.cme.msu.edu/) for tentative assignment of family, genus or species.

Further Analysis of Culture Purity

Agar plates of streaked bacterial cultures were further examined under magnification for assessment of purity. Alternative streaking techniques were developed for more effective separation of bacterial colonies. Incubation time of plates was increased from 48 hours to seven days to allow detection and isolation of slower growing bacteria in mixed cultures.

Results

Bacterial genomic DNA extracted using the GenElute Kit produced a single band of approximately 20 kbp on an agarose gel as below.

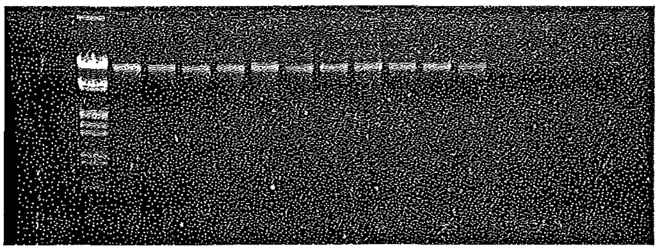


Figure 1. Agarose gel electrophoresis of bacterial gDNA using a Lambda DNA/ EcoRI-*Hind*III marker.

A single band of approximately 1500 bp was produced following 16S rDNA PCR amplification of the extracted DNA and no bands were evident in the water control as indicated below. A sufficient quantity of DNA was obtained from the PCR for sequencing.

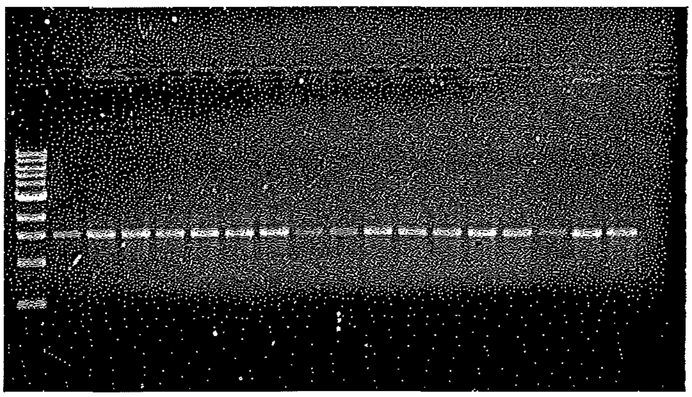


Figure 2. Agarose gel electrophoresis of 16S rDNA PCR products using a High mass ladder.

Results of sequence analysis are summarised in Table 1.

Table 1. Sequence Results form 16S rDNA PCR of purified genomic DNA.

Bacteria Accession No.	DNA Extraction	Identification	Sequence Quality
54	Colony	*Acinetobacter*	Good
54	Broth	*Acinetobacter*	Good
62	Colony	*Enterobacter*	Few Ns
62	Broth	*Enterobacteriacae*	More Ns than the broth extraction
68	Colony	*Acinetobacter*	Very good
68	Broth	*Acinetobacter*	Few Ns
69B	Colony	*Acinetobacter*	Very good
69B	Broth	*Acinetobacter*	Very good
70	Colony	*Enterobacteriacae*	Very good
76	Colony	*Klebsiella*	Very good
77	Broth	*Rhizobium*	Very good
101	Colony	*Rhizobium*	Very good
127A	Colony	*Acinetobacter*	Very good
127B	Colony	*Acinetobacter*	Very good
134	Colony	Undetermined	Too many Ns
154	Colony	*Micrococcus*	Few Ns

N: Ambiguous base call Good: Occasional N Very good: No or very few Ns

Examination of a number of bacterial cultures under magnification indicated that more than one type of bacteria was present. Improved streaking methods and longer lengths of plate incubation allowed separation of different bacteria in many mixed cultures. However in a number of mixed cultures, different bacteria were observed to be growing in very close association (a small colony growing one on top of another) and these bacteria were often not separable despite repeated streaking.

Discussion

Isolation of bacterial genomic DNA that ran as a single band on an agarose gel indicated that the DNA was of good integrity. The presence of a single band of expected size from 16S rDNA PCR and the absence of a band in the negative control indicated that the reaction was specific. In most instances, DNA sequence of very high quality was obtained from sequencing of PCR products. However in a number of cultures moderate to high levels of ambiguous bases were detected in 16S rDNA sequences. A possible explanation for the occurrence of ambiguous bases in DNA sequences is the presence of contaminants in the PCR reaction. Heterogeneity of rDNA sequences within the same bacterial strain may also contribute to the detection of ambiguous bases following sequencing (Garbeva *et.al.* 2004). Alternatively, unreadable sequence may indicate that cultures were not pure and contained more than one strain of bacteria (Thomas *et.al.* 2007). Further assessment of the purity of cultures by examination of cultures under magnification indicated that in a number of instances mixed cultures were present. While improved streaking methods and increased incubation times enabled the separation of different bacteria in many mixed cultures, certain bacteria that grew in close association were not able to be separated. Cloning and transformation experiments would be required to enable the identification of bacteria present in those mixed cultures, however this was beyond the scope of the current work. Interestingly, two bacteria isolated from the same culture (127) that had different morphologies were both identified as *Acinetobacter* species. This suggests that in certain cultures multiple strains of the same bacteria may be present. In other examples, bacterial colonies with markedly different morphologies were isolated from the same cultures. Identification of the different bacteria present in mixed cultures may indicate which species are compatible when combined, for example in research that aims to investigate growth benefits that might result when these bacteria are re-introduced to plants.

Optimisation of rapid methods for DNA template preparation for 16S rDNA PCR and sequencing of bacteria.

The use of crude cell lysates directly in the 16S rDNA PCR can obviate the need for more lengthy and expensive DNA extraction procedures. Such cell lysates have been prepared by boiling bacterial cells resuspended in an aqueous or alkali solutions. Variations in these methods have included the use of freeze thaw cycles and the addition of detergents such as SDS. The objective of this work was to optimise rapid procedures for the preparation of DNA templates for 16S rDNA PCR and sequencing.

Materials and Methods

Bacterial Cultures

Bacterial colonies were streaked onto nutrient agar and incubated at 28°C. All cultures were incubated for five to seven days following the first round of streaking. Purity of cultures was assessed under magnification. Streaking to obtain single colonies was repeated at least three times. In subsequent rounds of streaking, plates were incubated for one to seven days depending on the growth rate of the bacteria.

Preparation of Cell Lysates

Four methods were compared in preparation of cell lysates as follows:

A.1 Boiling of bacteria resuspended in TE

A bacterial colony was resuspended in 50μL of TE (10mM Tris HCl pH 7.4, 1mM EDTA pH 8.0) and heated to 99°C for 10 minutes. EDTA is used to chelate divalent cations required for enzyme activity in order to limit DNA degradation.

A.2 Boiling and freeze-thawing of bacteria resuspended in water

A bacterial colony was resupended in 50 μL sterile milliQ water and heated to 95 °C for 5 minutes. This suspension was subject to three freeze-thaw cycles (-80 °C for 15 min, 95 °C for 3 minutes). Cell debris was pelleted by centrifuging for 30 seconds and the supernatant was used directly in the PCR.

B.1 Lysis of bacteria with alkali and heat

A bacterial colony was resuspended in 50 μL of freshly prepared 0.2N NaOH in TE (10N NaOH was diluted 1:50 with sterile TE: 10mM Tris HCl pH 7.4, 1mM EDTA pH 8.0). The suspension was heated to 95°C for 10 minutes, allowed to cool and then centrifuged for 30 seconds. The supernatant was diluted 1:50 in TE for use in the 16S rDNA PCR reaction.

B.2 Lysis of bacteria with alkali, detergent and heat

A bacterial colony was resuspended in 50 μL of freshly prepared 0.05M NaOH amended with 0.25% SDS. This suspension was heated to 95°C for 10 minutes, allowed to cool and then centrifuged for 30 sec. The supernatant was diluted 1:50 in sterile MQ water for use in the 16S rDNA PCR reaction.

16S rDNA PCR
The 25µL PCR reaction consisted of 1X PCR Supermix (20mM Tris-HCl, 5mM KCL, 1.5mM, 200µM of each dNTP, 1 U Taq DNA polymerase; Invitrogen, California), 1µM of each primer (27F and 1492R Sigma-Genosys, Australia), 1.6 µL of cell lysate and an additional 0.5mM $MgCl_2$ to produce a final concentration of 2mM $MgCl_2$. PCR cycling and agarose gel electrophoresis were performed as described earlier.

Sequencing of PCR products
The PCR products were purified and then sequenced with an ABI 3730XL using ABI PRISM Big Dye Terminator Sequencing Chemistry (Version 3.1) by Macrogen Inc (Korea). The primer 926R (5'-CCGTCAATTCCTTTGAGTTT-3') was used for sequencing.

Sequence Analysis

Sequence chromatograms were inspected using the program Finch TV (www.geospiza.com/finchtv/index.htm). The Basic Local Alignment Search Tool (BLASTn) at the National Centre for Biotechnology Information (http://www.ncbi.nml.nih.gov/BLAST) was used to compare sequences (300-500bp) to those in GenBank. The distance tree produced by BLAST was used to infer the phylogenetic relationship of isolates with nearest neighbours (most closely related species). Sequences were also analysed with the SeqMatch and Classifier programs at the Ribosomal Database Project (http://rdp.cme.msu.edu/) for tentative assignment of genus.

Results and Discussion

Results of 16S rDNA PCR using several different methods of DNA template preparation are summarised in table below. The lysis procedure that used alkali, SDS and heat produced the most consistent results in 16S rDNA PCR. Where sufficient DNA was obtained for sequencing, the quality of sequence was very similar between different methods of template preparation.

No.	Method for DNA template preparation	16S PCR Result
A.1	Boiling of bacteria resuspended in TE	+/-
A.2	Boiling and freeze-thawing of bacteria resuspended in water	+
B.1	Lysis of bacteria with alkali and heat	++
B.2	Lysis of bacteria with alkali, detergent and heat	+++

+/- Faint band produced
+ Often inconsistent amplification was observed
++ Relatively consistent DNA amplification
+++ Most consistent DNA amplification

The variable results obtained from 16S PCR using different methods most likely indicated differential amounts of cell lysis produced. The method of preparation of cell lysates that used alkali, heat and SDS produced the most consistent results in 16S rDNA PCR . The quality of the DNA sequence obtained from PCR products prepared from extracted DNA and crude cell lysates was comparable. The method determined to be most efficient in terms of time, cost and reproducibility for 16S rDNA PCR and sequencing was the crude cell lysate prepared by heating a suspension of bacterial cells in an alkali solution containing SDS. This method was therefore used in subsequent research.

Identification of bacteria isolated from banana using 16S rDNA Sequencing

Introduction

Bacteria were isolated close to the growing point within banana suckers during indexing at tissue culture initiation. Since plant tissue culture relies on aseptic culture of plants there may be a need to re-inoculate cultures with beneficial microrganisms prior to planting into the rhizosphere that contains a wide range of diverse and sometimes problematic micro-organisms. This work begins the process of selecting and identifying those bacteria that show potential for further evaluation in banana. The aim is to identify bacteria that can provide significant benefits to banana production by improving plant growth with same inputs and/or improving plant defence against banana pathogens.

Material and Methods

The method determined to be most efficient in terms of time, cost and reproducibility for 16S rDNA PCR and sequencing used a crude cell lysate prepared by heating a suspension of bacterial cells in an alkali solution containing SDS. This method discussed in the previous section and described below was chosen for routine use. Optimised 16S rDNA sequencing procedures developed in previous chapter were used for the tentative identification of over one hundred bacteria that were recovered from meristem or shoot tip cultures.

Bacterial Cultures

1444 banana suckers were collected from Innisfail and Tully, North Queensland . All suckers were similar in size (ave. 250mm x 90mm) and cleaned to remove excess soil and tested negative for Banana Bunchy Top Virus, Cucumber Mosaic Virus, Banana Mild Mosaic Virus and Banana Streak Virus. Under aseptic conditions a tissue block containing the meristem was taken from each sucker, surface sterilised with 2.5% v/v sodium hypochlorite for 15 minutes, rinsed 3 times with sterile distilled water prior to excision of the meristem (diameter approx. 1mm) or the block of tissue containing the meristem for shoot-tip culture (25 mm x 10 mm). The banana plantlets were cultured as described by Hamill *et al.* (1992). Cultures were maintained on Murashige and Skoog medium (1962) containing 2.5 mg/l Benzyl amino purine and 1 mg/l Indoleacetic acid with 20g/l sucrose. Bacteria were isolated from the freshly excised meristems or shoot-tip cultures by streaking the base of the explant onto nutrient broth agar (beef extract, 3 g l^{-1}; peptone 5 g l^{-1}; sodium chloride, 8 g l^{-1}; yeast extract 2 g l^{-1}; with or without bacteriological agar, 15 g l^{-1}) and two tissue sections of approximately 1 mm^2 were extracted aseptically at the time of culture initiation adjacent to the meristem for meristem culture or mid section of the block of tissue for shoot-tip culture, visual bacteria contamination was also monitored in the newly initiated cultures as well as subsequent to culture establishment. Tissue sections were macerated with a sterile scalpel and inoculated to either nutrient broth or nutrient broth solidified with. Meristems (1mm) were cultured on Murashige and Skoog (1962) culture media. All cultures were incubated under tissue culture conditions of 28 ^{o}C, with nutrient broths shaken at 100 rpm.

Bacterial colonies were streaked onto nutrient agar and incubated at 28°C. All cultures were incubated for five to seven days following the first round of streaking. Purity of cultures was assessed under magnification. Streaking to obtain single colonies was repeated at least three times. In subsequent rounds of streaking, plates were incubated for one to seven days depending on the growth rate of the bacteria.

Preparation of cell lysate

A bacterial colony was resuspended in 50 μL of freshly prepared 0.05M NaOH amended with 0.25% SDS. This suspension was heated to 95°C for 10 minutes, allowed to cool and then centrifuged for 30 sec. The supernatant was diluted 1:50 in sterile MQ water.

16S rDNA PCR

The 50 μL PCR reaction consisted of 1X PCR Supermix (20mM Tris-HCl, 5mM KCL, 1.5mM, 200μM of each dNTP, 1 U Taq DNA polymerase; Invitrogen, California), 1μM of each primer (27F and 1492R Sigma-Genosys, Australia), 3.2 μL of cell lysate and an additional 0.5mM $MgCl_2$ to produce a final concentration of 2mM $MgCl_2$. Cycling conditions included an initial denaturation step at 94 °C for 4 min; 29 cycles of 94 °C for 45sec, 60 °C for 45 sec, 72 °C for 90sec; and a final extension at 72 °C for 10min. Cycling was performed in a PC 960G thermal cycler (Corbett Research, Australia).

Agarose Gel Electrophoresis

The gDNA was visualised by agarose gel electrophoresis with ethidium bromide staining. 5 μL of the PCR product was run on a 1% (wt/vol) agarose gel prepared with 1 X TAE and amended with 0.5 ug/ml of ethidium bromide. The gel was run with a Lambda DNA/ *Eco*RI-*Hin*dIII marker (MBI) in 1X TAE running buffer at 100V for 45 minutes.

Sequencing of PCR products

The PCR products were purified and then sequenced with an ABI 3730XL using ABI PRISM Big Dye Terminator Sequencing Chemistry (Version 3.1) by Macrogen Inc (Korea). The primer 926R (5'-CCGTCAATTCCTTTGAGTTT-3') was used for sequencing.

Sequence Analysis

Sequence chromatograms were inspected using the program Finch TV (www.geospiza.com/finchtv/index.htm). The Basic Local Alignment Search Tool (BLASTn) at the National Centre for Biotechnology Information (http://www.ncbi.nml.nih.gov/BLAST) was used to compare sequences (300-500bp) to those in GenBank. The distance tree produced by BLAST was used to infer the phylogenetic relationship of isolates with nearest neighbours (most closely related species). Sequences were also analysed with the SeqMatch and Classifier programs at the Ribosomal Database Project (http://rdp.cme.msu.edu/) for tentative assignment of genus.

Results and Discussion

1444 banana meristems were screened for bacteria. 256 of these banana suckers indexed positive to bacteria which resulted in 496 bacterial isolates since in some instances several bacteria were isolated from the one sucker. For identification purposes to date colonies were selected based on morphology as those that may be beneficial bacteria. There remain over two hundred bacterial isolated to be identified. The results of 16S rDNA sequence analysis of bacteria are indicated in the table below.

Identification	Activity/Characteristics of this type of bacteria	Number Isolated	Percent %
***Acidovorax* sp.**	**Nitrogen fixation; Bioremediation;** Some plant pathogenic species	3	1.97
Acinetobacter* sp./*Acinetobacter baylyi	Occur in soil, water and sewerage	10	6.58
Acinetobacter juni	Soil, water, skin; Human pathogenic strains	1	0.66
Ancylobacter*/unclassified *Hyphomicrobiaceae	Found in soil and water	1	0.66
***Arthrobacter* sp.**	Ubiquitous bacteria, often found in soil and the rhizosphere	2	1.32
Bacillus megaterium/Bacillus flexus	**Disease resistance, phosphate solubilisation**	14	9.21
Bacillus pumulis	**Disease resistance, ISR, growth promotion**	12	7.89
Bacillus cereus/ B.thuringeniensis/B.anthracis	**Disease resistance, insecticidal,** Human pathogenic strains	3	1.97
Bacillus aquimaris/ Bacillus marisflavi	**Sea water, rhizosphere; Related to nitrogen fixing bacteria**	1	0.66
***Brevibacterium* sp.**	Human skin and in dairy products; Human pathogenic strains	1	0.66
***Comamonas* sp.**	Rhizosphere bacteria; **Bioremediation**	1	0.66
***Corynebacterium* sp.**	Plant and human pathogenic strains	1	0.66
***Devosia* sp.**	Soil and rhizosphere bacteria; **Legume root nodulation**	1	0.66
Klebsiella/Enterobacter/Serratia	**Plant growth promotion, disease resistance**, human pathogenic	16	10.53
Pantoea ananatis/Pantoea agglomerans	**Plant growth promotion, disease resistance,** human pathogenic	5	3.29
Unclassified *Enterobacteriaceae*		7	4.61
Herbaspirillum seropedicae/H.rubrisubalbicans	**Nitrogen fixation, plant growth promotion**	6	3.95
Unclassified *Herbaspirillum*		9	5.92
Microbacterium sp.	Found on skin, soil, water, rhizosphere. **disease resistance**	9	0.66
Mycobacteium sp.	Human pathogenic	1	0.66

Nocardia sp.	Human pathogenic	1	5.92
***Paenibacillus* sp.**	**Mycorrhizal helper bacteria, plant growth promotion**	1	0.66
Pseudomonas putida/ Pseudomonas fulva	**Disease resistance, ISR, plant growth promotion**	9	5.92
Pseudomonas pseudoalcaligenes/P.alcaliphila	Rhizosphere bacteria, **bioremediation**	4	2.63
Rhizobium radiobacter/Ragrobacterium	**Legume nodulation, plant growth promotion of non-legumes**	22	14.47
Stenotrophomonas maltophilia	**Plant growth promotion,** disease resistance, human pathogen	2	1.32
***Streptomyces* sp.**	**Disease resistance;** Some species are plant pathogenic	3	1.97
***Variovorax* sp.**	**Soil, rhizosphere bacterium; Degrade bioplastic, pesticides**	3	1.97
***Xanthomonas* sp.**	Rhizosphere bacterium; Some species are plant pathogenic	3	1.97
Total potentially beneficial	**Identified using 16S sequencing**	**124**	**81.6%**
Total identified	**Identified using 16S sequencing**	**152**	**100.00%**
Unknown	**Yet to be identified**	**220**	

DNA sequence analysis indicated that diverse populations of endophytic bacteria are found inside banana suckers Of the 152 bacterial isolates screened so far 82% have been described to provide benefits to plants. A high percentage of these bacteria have been reported to have plant growth promotion and/or disease reducing capabilities. For example Enterobacteria have frequently been isolated from the rhizosphere and endophytic locations; nitrogen fixing ability, growth promotion and antagonism of plant pathogens. Many of these bacteria are nitrogen fixing organisms with the potential to reduce the requirement for applied synthetic fertilisers, that has potential benefits in terms of soil health, reduced costs and reduced emissions of greenhouse gases.

This is promising for further research into improving banana growth and plant defence against diseases such as Fusarium wilt. Plant defence activation is required in banana against several important diseases. For example banana plantlets produced from tissue culture show reduced tolerance to Fuarium wilt compared to bits or suckers when planted in infected fields. The plant tissue culture system eliminates all micro-organisms including beneficial rhizosphere bacteria so it is likely that if beneficial bacteria could be re-established within the banana plants they will assist them to improve growth and defend against plant pathogens. Future work will aim to investigate beneficial properties of these bacteria in banana and then develop ways to re-inoculate plants either *in vitro* or at the deflasking phase of acclimatisation. Future work in this area offers ways for growers to improve sustainable production practices.

Technology transfer

Industry has been kept informed on progress in banana tissue culture research through most available media.

The main conduit to growers has been through regular publication of articles in the industry journal Australian Bananas. Papers and posters have been presented at every Australian banana congress.

Sharon Hamill was a member of the organising committee of the 7th Australian Banana Industry Congress.

Tissue culture problem solving has been provided to commercial laboratories and nurseries on request and on site training was provided to four commercial QBAN laboratories as requested.

Research has been published in peer reviewed journals.

Ongoing consultation with biosecurity resulted in development in draft ICA although this has not been implemented.

Growers Inspect Trial Progress

On Friday 15 April a handful of banana growers braved intermittent showers to inspect progress in the evaluation of a new Cavendish variety at South Johnstone Research Station. The replicated trial includes the new selection Formosana, from Taiwan, compared alongside the industry standards of Williams and Grande Naine. Participants were able to see first hand the very large plant crop bunches of Formosana that were hanging as well as do a taste comparison of Formosana with Williams – the verdict? The growers thought it tasted much the same as Williams. As well some preliminary results from the South Johnstone trial were presented.

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Recommendations

The industry has faced numerous challenges over the past three years including the impact of C yclone Larry and the ongoing threat of imports. 2001-2004 saw the industry contract due to uncertainty and reduced profit margins. 2004-2007 has seen the industry struggle to regain a focus on the future. Industry value has increased along with this positive momentum and more growers are now implementing research outcomes for significant gains to profitability. The increase has been significant, in 2002- 2004 the gross value of Queensland production remained relatively static at $285 million (QDPI prospects forecast Dec 2004), 2006-07 gross value was $390 million and 2007-08 is forecast to increase by 13% to $450 million dollars (Prospects for Queensland Industries 2007-08 (Department of Primary Industries and Fisheries 2007). Growers are now moving to adopt technologies that have been developed to improve profits. For example 2004-2007 has seen significant increase in use of tissue culture for planting material. In 2007 over 30% of commercial planting material was of tissue culture origin.

With a majority vote to a National Banana levy, industry is now in the process of developing a new future strategic plan and direction. Industry recognise the important role that research contributes to improved efficiencies and profits including prevention and management of emergent issues such as pests and diseases. Over the last few years regulatory processes and systems have been changing and now must be reviewed and improved. Most states generally no longer provide plant health staff dedicated to the banana industry as was the case previously. Now industry is increasingly tasked with management of diseases and development of solutions. With the dissolution of the Queensland Banana Industry Protection Board many plant health responsibilities will now fall to the Australian Banana Growers Council, the newly elected national banana body. With this comes urgent need to redevelop previous practices such as the case with the Quality Banana Approved Nursery scheme which is now facing a time of expansion.

With ongoing industry expansion there is also emerging a problem in allocation of sufficient resources needed to manage and regulate the Australian banana industry. One of the challenges of the future will be to develop banana plant health plans and strategies appropriate for existing or emerging pests in diseases for each banana producing state while supporting the research needed to do this as well as provide improvements for industry.

However with these ongoing changes in responsibility and challenges from pest and diseases, imports and environment also comes with opportunities. Research to improve banana industry practices remain of great importance and the Australian industry must take advantage of research outcomes. Growers can maintain forward momentum, remain proactive and move to adopt new technologies, processes and practices that will refine and improve production systems. By supporting and embracing research in the future growers will be able to both improve profits and reduce inputs and minimise impacts on the environment.

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