The fate of genetically modified yeast in the environment

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

Considerable efforts have been made to improve strains of the wine yeast *Saccharomyces cerevisiae* through the use of genetic engineering. Although the process is well defined, globally there is much resistance towards the use of genetically modified organisms (GMOs), primarily because little is known about their environmental fate and their potential effect on naturally occurring organisms. The public concern is mainly focused on the uncertainty associated with the impact of the deliberate or accidental release of a GMO into the environment. As a consequence, there is an urgent need to assess the potential risks involved with the use of this new technology. For the eventual global acceptance of any GMO, it is imperative that the consumer must be convinced that it is ultimately safe for human consumption and the environment. In order to achieve this, certain risk assessment procedures must be performed on each and every GMO that is planned to be released into the environment. Although some of the genetically modified (GM) yeasts that have been developed comply with the strict legislation of most countries and have been cleared by regulatory authorities for commercial use, GM yeasts have not, as yet, been used for the commercial production of GM bread, beer or wine. Nevertheless, the use of GM yeasts in the market appears imminent and there is an urgent need to assess and address the perceived health and environmental risks associated with GM foods.

The overall objective of this research was to evaluate key environmental issues concerning the use of GM yeasts. The focus was on comparing the behaviour of specific parental and GM yeast strains in model systems in order to determine whether the GM strains may have any selective advantage, which could lead to their spreading. Specifically, it involved monitoring of the growth behaviour of selected GM yeasts within a vineyard microbial community and in fermentations, as well as the interaction of these yeasts with sand and glass surfaces in an aqueous environment. The GM yeasts under investigation were recombinant strains of a well-known, industrial strain of *S. cerevisiae* VIN13 expressing an α-amylase (designated GMY1); an endo-β-1,4-glucanase and endo-β-xylanase (designated GMY2); and a pectate lyase and polygalacturonase (designated GMY3).

The GM yeasts were mist-inoculated onto individually-contained blocks consisting of one-year old grapevines in a secluded glasshouse environment. Specifically, the numbers and dynamics of GM yeast survival, as well as the effect of an introduced GM yeast on the yeast community dynamics and numbers, were investigated. Overall, it was found that the most prevalent wild yeasts isolated from the grapevines were *Rhodotorula, Yarrowia lipolytica, Pichia* and *Candida* spp. VIN13 and the GM yeasts did not affect the overall ecological balance of the microflora on the grapevines. Wild strains of *S. cerevisiae* were seldom isolated from the grapevines. With a few exceptions, the overall detection of GM yeasts was numerically limited. Co-inoculation of (VIN13+GMY1) and (GMY1+GMY2) revealed

detection approximately in the same ratio at which they were inoculated, with small differences in the order of GMY2>GMY1>GMY3. GM yeasts were rarely isolated from bark and soil samples. Spontaneous fermentation of the grapes harvested from the different treated blocks indicated that the GM yeasts survived on the berries, that the natural fermenting ability of VIN13 was conserved in the recombinant strains, and that the GM yeasts did not have any competitive advantage.

The soil environment forms an important part of the biosphere and the transport and attenuation of a GM yeast in this matrix will to a large extent affect their ultimate fate in the environment. In soil, microorganisms either occur as suspended cells in pore water or as biofilms on soil surfaces. Although less extensive than a typical soil yeast, *Cryptococcus,* epifluorescent staining of biofilms confirmed that VIN13 and GMY1 were capable of existing in a biofilm mode on sand granules and glass. Data on effluent numbers detected in flow cells indicated that GMY1 had no advantage due to the genetic modification and had the same reproductive success as VIN13. These strains either had no difference in biofilm density or GMY1 was less dense than VIN13. When co-inoculated, GMY1 had no negative influence on the mobility of *Cryptococcus* through a sand column, as well as the ability of *Cryptococcus* to form biofilms. Furthermore, it was found that GMY1 did not incorporate well into a stable biofilm community on glass, but did not disrupt the biofilm community either.

This is the first report of the assessment of the fate of GM strains of VIN13 that are suitable for the wine and baking industry. The investigation of the GM yeasts in this study under different scenarios is a good start to an extensive and necessary risk assessment procedure for the possible use of these GM yeasts in the industry. This study could lead to the provision of much-needed scientific and technical information to both industry and regulating bodies. The outcome of this research is also intended to serve as a basis for information sharing with public interest groups.

OPSOMMING

Aansienlike pogings is reeds aangewend om rasse van die wyngis, *Saccharomyces cerevisiae,* deur middel van genetiese manipulering te verbeter. Alhoewel hierdie proses goed gedefinieerd is, is daar wêreldwyd heelwat teenkanting teen die gebruik van geneties gemanipuleerde organismes (GMO's). Dit is hoofsaaklik te wyte daaraan dat so min bekend is oor hul lot in die omgewing en hul potensiële effek op die organismes wat natuurlik voorkom. Die publiek is veral besorg oor die onsekerheid verbonde aan die bestemde of toevallige vrylating van 'n GMO in die omgewing. Gevolglik is daar 'n dringende behoefte om die potensiële risiko's in die gebruik van hierdie nuwe tegnologie te bepaal. Dit is van uiterste belang dat die verbruiker oortuig sal word van die veiligheid vir menslike gebruik en die omgewing voordat enige GMO uiteindelik wêreldwyd aanvaarbaar sal word. Om dit te kan bereik sal sekere risiko-bepalende prosedures toegepas moet word op ieder en elke GMO wat beplan word om vry gelaat te word in die omgewing. Alhoewel sommige van die geneties gemanipuleerde (GM) giste aan die streng wetgewing van die meeste lande voldoen en deur die owerhede vir kommersiële gebruik goedgekeur is, word GM-giste nog steeds nie vir die produksie van GM brood, bier of wyn gebruik nie. Ten spyte hiervan, blyk die gebruik van GM-giste onafwendbaar te wees en is daar dus 'n dringende behoefte om die voorspelde gesondheids- en omgewingsrisiko's wat met GM voedsel geassosieer word, aan te spreek.

Die oorhoofse doel van hierdie navorsing was om belangrike omgewingskwessies aangaande die gebruik van GM-giste te evalueer. Die fokus was op die vergelyking van die gedrag van spesifieke oorspronklike gisrasse en GM-gisrasse in modelsisteme sodat daar bepaal kon word of die GM-gisrasse enige selektiewe voordele het wat moontlik tot hul onbeheerde verspreiding in die natuur sou kon lei. Dit het spesifiek die monitering van die groei van geselekteerde GM-giste binne 'n mikrobiese gemeenskap op wingerd en in fermentasies behels, asook die interaksie van hierdie giste met grond en glas oppervlaktes in 'n wateromgewing. Die GM-giste wat in hierdie studie gebruik is, was rekombinante rasse van 'n bekende industriële ras van *S. cerevisiae,* VIN13, wat geneties gemodifiseerd was om 'n α-amylase (aangedui as GMG1); 'n endo-β-1,4-glukanase en 'n endo-β-xilanase (aangedui as GMG2); en 'n pektaatliase en 'n poligalaktorinase (aangedui as GMG3) uit te druk. Die GM-giste is op afsonderlike blokke van eenjaaroue wingerdplante binne-in 'n beskutte kweekhuis gesproei-inokuleer. Daar was spesifiek na die selgetalle en dinamika van die oorlewende GM-giste gelet, asook wat die invloed was van die inokulasie van 'n GM gis op die selgetalle van die natuurlike gisgemeenskap. Daar is bevind dat die wildegiste *Rhodotorula, Yarrowia lipolytica, Pichia* en *Candida* spp die gereeldste van die wingerd geïsoleer is. VIN13 en die GM-giste het nie die ekologiese balans van die natuurlike mikrobiese populasie op die wingerd versteur nie. Wilde rasse van *S. cerevisiae* is selde geïsoleer vanaf die wingerd. In die meeste gevalle is daar bevind dat wanneer

GM-giste opgespoor is, hulle in lae selgetalle voorgekom het. Waar giste saam geïnokuleer was, was die opsporing van (VIN13+GMY1) en (GMY1+GMY2) ongeveer in dieselfde verhouding as waarin hul geïnokuleer was, terwyl klein verskille in die orde van GMY2>GMY1>GMY3 opgemerk is. GM-giste is selde vanaf bas- en grond-monsters geïsoleer. Spontane fermentasies van druiwe wat geoes vanaf die verskillende behandelde blokke is, het daarop gedui dat die GM-giste wel op die druiwe oorleef, dat die natuurlike vermoë van VIN13 om te kan fermenteer in die gemodifiseerde gisrasse behoue gebly het en dat die GM-giste geensins deur die genetiese modifikasies bevoordeel was nie.

Grond is 'n belangrike deel van die biosfeer en die verspreiding en aanhegting van 'n GM-gis in hierdie matriks sal sy algehele lot in die omgewing tot 'n groot mate beïnvloed. In die grond kom mikroörganismes as gesuspendeerde selle in poriewater of as biofilms op die oppervlaktes van grond voor. Alhoewel biofilmvorming van VIN13 en GMG1 swakker was as in die geval van 'n tipiese grondgis, *Cryptococcus,* het epifluoresserende kleuring van hierdie *S. cerevisiae*-giste bevestig dat VIN13 en GMG1 in staat was om as biofilms op sandkorrels en glas te oorleef. Gebaseer op seltellings in vloeiseluitlaat, kon daar afgelei word dat GMG1 geen selektiewe voordeel geniet het as gevolg van die genetiese modifikasie nie en dat die gis net so reproduktief was as VIN13. Hierdie gisrasse het geen verskil in biofilmdigtheid getoon nie of die biofilmvorming van GMG1 was minder dig as die van VIN13. Wanneer GMG1 saam met *Cryptococcus* geïnokuleer was, het GMG1 geen negatiewe invloed op die beweeglikheid van *Cryptococcus* deur 'n sandkolom gehad nie en die vermoë van *Cryptococcus* om biofilms te vorm is ook nie beïnvloed nie. Daar is verder ook bevind dat GMG1 nie goed binne-in 'n gestabiliseerde biofilmgemeenskap op glas geïnkorporeer het nie, maar dat die gis ook nie die biofilmgemeenskap versteur het nie.

Hierdie studie verteenwoordig die eerste ondersoek ooit oor die lot, oorlewing en groeigedrag van GM-wyngiste in biologies-afgesonderde wingerd-, fermentasie-, modelgrond- en modelwater-ekosisteme. Die bestudering van hierdie GM-giste onder verskillende omgewingstoestande in afgeslote ekosisteme lê 'n stewige basis vir verdere ondersoeke en die ontwikkeling van omvattende en noodsaaklike risikobepalingsprosedures betreffende die moontlike toekomstige gebruik van GM-giste in die industrie. Hierdie studie baan die weg tot die verkryging van noodsaaklike wetenskaplike en tegniese inligting oor die veiligheidsaspekte rakende GM-wyngiste en dit kan van groot waarde vir die industrie, owerhede en verbruikerspubliek wees.

This dissertation is dedicated to my best friend, Gawie Toerien.
Hierdie proefskrif is opgedra aan my beste vriend, Gawie Toerien.

We should use science to make us "masters and possessors of nature".
Descartes

BIOGRAPHICAL SKETCH

Heidi Schoeman was born in Germiston, South Africa on the 5th of March 1974. She matriculated with distinction at Eben Dönges High School, Kraaifontein in 1991. Heidi enrolled at the University of Stellenbosch in 1992 and obtained a BScAgric degree, majoring in Microbiology and Biochemistry, in 1995. She followed with her MScAgric degree in Microbiology in 1998 and enrolled for her PhDAgric degree in 1999.

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PREFACE

This dissertation is presented as a compilation of five chapters. Each chapter is introduced separately.

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

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Environmental, societal and regulatory issues concerning genetically modified organisms

Chapter 3 **RESEARCH RESULTS**
The detection and monitoring of genetically modified yeasts within a vineyard microbial population

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The detection and monitoring of genetically modified yeasts within a vineyard microbial population

CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

In the past decade, biotechnological advances have increased enormously. A considerable part of many of these advances involves genetic engineering. In the early 1970s, the first genetically modified organisms (GMOs) were created through recombinant DNA technology. Genetically modified (GM) plants were always at the forefront and in 1983 the first of these plants was produced (Hails and Kinderlerer, 2003). The global surface area cultivated with GM crops totalled 67.7 million hectares in 2003, indicating an increase of 15% since 2002 (International Service for the Acquisition of Agri-biotech Applications, 2003).

In response to a highly competitive wine market, much work has already been done on the improvement of yeast strains through genetic engineering (Pretorius, 2000). Although this process is well defined, globally there is much resistance to the use of GMOs. The public concern is mainly focused on the uncertainty associated with the impact of the deliberate or accidental release of a GMO into the environment. Little is known about the environmental fate of GMOs and their potential effect on naturally occurring organisms. It has been stated that critics of GMOs are often motivated by political agendas and the protection of agricultural markets and, in the process, alarm the public (Pretorius, 2000). Public interest groups and non-governmental organisations also feel excluded from the debate on the use of GMOs and do not trust those working with them. Therefore, there is a growing coalition calling for a moratorium on the commercialisation of GMOs and products derived from them. It is clear that there is a strong need for consistent, well-defined and scientifically based guidelines for regulation. It is of strategic importance to the South African wine industry to proactively consider this question, primarily to remain competitive in the global economy, but also to address public concerns.

Many strains of *Saccharomyces cerevisiae* has already been genetically tailored for the production of a wide variety of products, including heterologous proteins (Pretorius, 2000). Compelling evidence that demonstrates the safety of GMOs for human consumption and the environment is required before the use of GMOs will be generally (globally) accepted. In order to achieve this, certain risk assessment procedures must be performed on each and every GMO that is planned to be released into the environment.

The study of fungal biofilms is still in its infancy; in contrast to the extensive literature describing bacterial biofilms (see O'Toole *et al.*, 1999, 2000; Watnick and Kolter, 2000 for excellent reviews). Therefore, studies of *S. cerevisiae* in model systems that incorporate the biofilm mode of existence may potentially reveal valuable information in terms its behaviour in the environment.

The overall objective of this study was to evaluate key environmental issues concerning the use of GMOs, such as GM yeast. Specifically, three GM strains of the industrial wine yeast *S. cerevisiae* VIN13 were investigated. These included VIN13 expressing *LKA1*, which encodes an α-amylase; VIN13 expressing two genes, namely *end1* (encoding endo-β-1,4-glucanase) and *XYNC* (encoding endo-β-xylanase); and VIN13 expressing two genes, namely *pelE* (encoding pectate lyase) and *peh1* (encoding polygalacturonase). The main focus was on the amylolytic *S. cerevisiae,* designated GMY1, where a null hypothesis was investigated that states that no difference exists between the ecological behaviour of the wild-type strain, VIN13, and GMY1. This hypothesis was specifically tested on grapevines (including grapes, leaves and bark), in fermentations and in the interaction of these yeasts with sand and glass surfaces in an aqueous environment.

The overall aim of this research was to establish a pool of scientifically derived information and expertise that will contribute to the understanding of GM yeast and, in particular, their fate and effect in the environment. The design of this research study did not replicate any previous study and is entirely unique.

The specific objectives of this study were:

1. To assess the fate of a few chosen GM yeasts when released into a secluded glasshouse environment containing grapevines. Specifically, the numbers and dynamics of GM yeast survival, as well as the effect of an introduced GM yeast on the yeast community dynamics and numbers, were investigated. The detection and monitoring of the GM yeasts within a vineyard microbial community started in 1999, with the seasonal monitoring (May to October) of the basal wild yeast population on Riesling grapevines. Different combinations of GM yeasts were then inoculated onto the grapevines in 2000 and 2001 and their detection was monitored by means of a classical approach.
2. To assess the behaviour of GM yeasts within a fermentation environment. Spontaneous fermentations were performed with berries harvested from the glasshouse. In additional controlled fermentations, yeast strains were inoculated in the same combinations as on the grapevines.
3. To assess the fate of GMY1 when released into model systems that simulate the physical and biotic controls present in the environment. This included an aqueous environment in which biofilm experiments were employed and a soil environment in which GM yeast behaviour in soil columns was investigated. In the soil environment, the transport and attenuation of GMY1 in the soil matrix, particularly in the pore water and in the colonisation of soil surfaces, were investigated.

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CHAPTER 2

LITERATURE REVIEW

Environmental, societal and regulatory issues concerning genetically modified organisms

2. LITERATURE REVIEW

2.1 INTRODUCTION

When the science of recombinant DNA technology emerged in the 1970s, critics and politicians feared that science was proceeding too far, too fast and that we were not really in control of these technologies. However, what arose from research laboratories were many life-saving and life-enhancing medications and vaccines. It started off with the production of recombinant insulin in 1982, followed by human growth hormone, clotting factors for haemophiliacs, fertility drugs, erythropoietin and many other pharmaceuticals (Feldbaum, 2002). According to one of the most comprehensive databases (http://www.icgeb.trieste.it/~bsafesrv/), the last decade (1990-2000) has seen the number of biosafety-related publications on transgenic organisms increase significantly (more than 2500 citations).

Until now, most effort in genetic engineering has focused on the modification of crops, although a significant effort has also been made to develop non-crop-related agricultural food applications, e.g. animal vaccines, hormones, fish, food animals, active food ingredients and microorganisms (Phillips, 2002).

The impact of biotechnology in the food industry ranges from the genetic modification of raw materials, such as wheat and other cereals, lactic acid bacteria and yeast, to applications in the preservation of raw materials, the production of food additives (e.g. enzymes) and bioprocessing itself (Linko *et al.*, 1997). The application of genetic manipulation of microorganisms for the enhancement of enzyme production has had the most extensive use in food manufacturing (Barling *et al.*, 1999). The advantages of the application of genetic modification include, amongst other things, lower production costs, environmental benefits and the many possibilities of developing products with better sensory, convenience and health properties (Grunert *et al.*, 2001).

Since wine is not an essential food, the wine industry has responded late to the threat or promise of gene technology. Instead, pioneering biotechnological companies were of the opinion that research into annual, subsistence crops, such as maize, would harvest quicker profits. In addition, research into grapevines is a slow process, because they are woody perennials. However, the wine industry is set for potentially irreversible changes once the market perception of genetically modified (GM) wine-making products changes (Waldin, 2001).

Two opposite possible future scenarios exist for biotechnology. On the one hand, some proponents and many agricultural food policy makers around the world propose the future of biotechnology as limitless in all the beneficial applications, in which food shortages are overcome, the environment is improved, diseases are healed or eliminated and society becomes healthy and prosperous. On the other hand, a smaller but significant number of policy makers, consumers and members of society see the future as one where biotechnology will increase food insecurity,

threaten the environment and human health and eventually impoverish some parts of society (Phillips, 2002).

Although many experts disregard the possibility of serious risks arising from the application of genetic engineering (Scholderer *et al.*, 1998), agricultural biotechnology has become the scapegoat for several difficult socio-economic issues of which globalisation, liberalisation and a loss of local tradition and ethical values are just a few (Braun, 2002). In the heated debate on GM food, the media has often presented it as something in terms of which the whole concept is more important than the scientific reality relating to it.

So far, the public's perception of the risks of GM food has overshadowed its view of the possible benefits. A significant part of the public also still believes that GM food will be proven unhealthy in the end and that the spreading of genetically manipulated organisms (GMOs) into the environment will cause a loss of biodiversity (Pretorius, 2000) and an increase in allergenicity and antibiotic resistance (Gaskell *et al.*, 1999). There is also doubt among the public that sufficient legal and practical protection exist against the occurrence of accidents involving GMOs (Viviers and Pretorius, 2002). According to a relatively recent European study, public concerns are not really about the risks of GM technology *per se*, but have more to do with trusting the bodies that are responsible for its regulation (Marris *et al.*, 2002). It seems that the specific type and area of GM application also strongly influence the consumer's attitude towards this technology. The genetic engineering of plants and microorganisms, as well as applications in the medical industry, appears to be more acceptable than the engineering of animals and applications in the food industry (Frewer *et al.*, 1997).

Improving public acceptance of GM food became more difficult after the commercial application of GM plants (Falk *et al.*, 2002; Harlander, 2002; Hino, 2002; Moseley, 2002). Much of the food that is consumed has been or is continually being genetically engineered by natural processes that do not differ in any fundamental way from the current GM technology (Trewavas, 2000). Many important food plants are so-called introduced species, e.g. Canola and many barley varieties, and the unnatural means by which they have been developed have long been accepted as an acceptable part of our technical advancement.

What is a GMO? A general definition of GMOs states that they are organisms in which the genetic material has been changed in a way that does not occur naturally by mutation, mating or natural recombination. If genetic engineering allows for individual genes to be carried over from one organism to another, even between non-related species, the relevance of this definition can be speculated on in the light of current scientific knowledge, particularly in the field of genomics. What is the safest means of strain improvement, considering that safety issues should predominate in the GMO debate? One of the issues dividing people in the GMO debate is whether the final construction or the way in which the modification was performed should be considered. This can be strongly debated if it is taken into account that, in principle,

a mutation could be obtained in different ways, e.g. spontaneous mutation, induced mutagenesis or genetic engineering. If recombinant DNA technology is used to introduce a gene deletion into an organism, even if this mutation was similar to what might arise naturally, such an organism would still be considered a GMO (Renault, 2002). As will be elaborated on later, this is a key point of difference between the regulation systems in the European Union (EU) and the United States of America (USA). However, there seems to be a growing consensus that risk is primarily a function of the characteristics of a product and not of the use of genetic engineering *per se* (Pretorius, 2000).

The eventual acceptance of GMOs will rely to a large extent on the acceptance of GM plants and their products. The acceptance of GM microorganisms will therefore indirectly be influenced by the public's perception and acceptance of GM plants and food. In this overview on GMOs, a general approach is taken that includes reference to GM plants, but with special emphasis on GM microorganisms and, in particular, the industrial yeast, *Saccharomyces cerevisiae*.

2.2 THE IMPLICATIONS OF GMOs AND GENE TRANSFER

2.2.1 WHAT IS THE SIGNIFICANCE OF GENE TRANSFER?

One of the biggest fears regarding the safety of GMOs relates to what would happen if transgenes should escape into other species after their release into the environment or following consumption by humans or other animals, and cause undesirable, uncontrollable effects. Considering that in nature organisms from different species do transfer genes to each other, this is a legitimate concern. The real question or concern is thus not related to whether transgenes will move, because they possibly will, but whether the movement of transgenes will be more dangerous than the natural transfer of genes between species (Holmes, 2002).

There is strong evidence that suggests that the GMOs of today do not pose such a threat. However, it is possible that new transgenes in the future could carry a greater potential for harm. The riskier transgenes could, for example, be ones that cause plants to increase in size, be healthier, or anything that would make them expand their range of habitat, e.g. the development of cold, drought or salt tolerance. Changes like these could have major ecological consequences if the wild relatives of these plants obtain the transgenes (Holmes, 2002).

In nature however, very few genes take great evolutionary leaps between unrelated species. The transfer of genes between plants and animals, plants and bacteria and animals and bacteria rarely occurs (Holmes, 2002).

Gene flow *per se* does not necessarily have to be a problem. If a transgene increases the chance of survival of a recipient organism relative to that of its wild counterpart, the gene will spread throughout the wild population. If survival is not more likely for the organism, it is only a trait with no persistency. This can, for example, also be applied to microorganisms that have been genetically engineered

to degrade toxic waste. The specialised enzymes become a metabolic burden once the toxic waste is degraded and the microbe will most probably fade away (Holmes, 2002).

2.2.2 MICROBIAL GENE TRANSFER AND THE ANTIBIOTIC RESISTANCE SCARE

Vertical gene transfer in bacteria occurs through the transmission of genetic material from one generation to the next via asexual reproduction. Bacteria compensate for the absence of the genetic diversity that is acquired through sexual reproduction by utilising several mechanisms that allow horizontal gene transfer to occur (Syvanen, 1994). In a natural environment, these mechanisms of DNA transfer between microorganisms include conjugation, transformation of released DNA and bacteriophage-mediated transduction (Gasson, 2000).

Bacteria are true experts in horizontal gene transfer, as can be seen from the explosion of antibiotic resistance over the last few decades, during which a wide range of species have exchanged resistance genes (Holmes, 2002). Due to the misuse of antibiotics in agriculture and medicine, researchers have found that antibiotic resistance genes are already widespread in bacteria from animal digestive tracts (Coghlan, 2002). Bacteria have highly evolved ways for the acquisition and rearrangement of genetic material - a mechanism for horizontal gene flow and DNA rearrangement in their evolution. The issue of gene transfer in the GMO debate became more charged when antibiotic resistance genes were included in some GM plants intended for release. Questions arose about the existence of mechanisms by which transgenes may be acquired by bacteria and other microorganisms (Gasson, 2000).

Since the first genetic engineering experiments were carried out in 1984, not a single case of a "runaway" GMO has been reported. The main reason for this is that bacteria expressing foreign genes experience these transgenes as a genetic burden. Any transgene will only be maintained if it provides an advantage to the host organism. Therefore, antibiotic resistance genes will only be retained by an organism if it encounters that particular antibiotic in its environment (Thomson, 2002). The major problem in the antibiotic resistance scare is perhaps not gene transfer *per se*, but the overuse and misuse of antibiotics. Regardless of how many different genes for antibiotic resistance is used in recombinant DNA technology, if the selective pressure (i.e. the antibiotics) is provided, multi-resistant strains will eventually be encountered.

Nevertheless, the issue of the transfer of antibiotic resistant genes remains important and will be elaborated on further in this overview. The only way in which this transfer can be avoided in the future is if the transformation techniques for all kinds of modified organisms are adjusted in such a way that no unnecessary foreign DNA will remain in GMOs.

2.2.3 THE FATE OF INGESTED DNA

A valid consideration in this debate is whether naked DNA survives in the digestive system and is able to cause transfer events. The sensitivity of DNA to inactivation and degradation is one of the major factors preventing the transfer of DNA. Particularly when DNA is ingested via food and feed, the deoxyribonuclease I produced by the salivary glands, pancreas and the small intestine acts as a potent degrading agent. The low pH found in the stomach or ruminant abomasal also contributes to eliminating biological activity of DNA (Beever and Kemp, 2000).

Gasson (2000) highlighted a few studies on the survival of DNA that add to our understanding of the rate at which ingested DNA is destroyed through natural processes. These studies indicate that, although DNA may be available for transformation in the oral cavity, it is rapidly inactivated further down the digestive tract. Mercer *et al.* (1999) specifically tested the effect of human saliva on the survival of DNA. Competitive polymerase chain reaction (PCR) was combined with transformation into the naturally occurring oral bacterium, *Streptococcus gordonii,* to test for biological activity. Enough biologically active DNA survived to generate transformants, although the frequency was low. In another study, Duggan *et al.* (2000) evaluated the degradation of DNA by ovine saliva, rumen fluid and silage effluent. The biological activity was measured by *Escherichia coli* transformation. After the DNA had been exposed to rumen fluid or silage effluent for half an hour, it was still possible to perform PCR on the DNA, although its ability to transform was lost within one minute. In contrast, the transforming ability was not lost after 24 hours (h) of exposure to saliva.

An experiment was conducted by researchers at Britain's Food Standards Agency to determine if any genes could be transferred from GM food to microorganisms in the human digestive tract. Twelve healthy volunteers and seven volunteers whose colons had been removed surgically were fed burgers and milkshake containing GM soya. The GM soya expressed an extra gene conferring resistance to the herbicide, glyphosate. The stools of both groups of volunteers were examined and no trace of soya DNA was found in the volunteers whose digestive systems were intact. However, the stools of the volunteers with ileostomy bags showed that up to 3.7% of the soya DNA remained in the bags. After bacteria from these samples were grown, trace amounts of GM DNA could be detected, which suggests that only a very few bacteria had taken up a transgene from the GM food. It was concluded that, although some of the DNA might survive as far as the small intestine, it is all broken down on the way through the colon in people with normal digestive systems. Even if DNA is taken up by some bacteria, it will not survive the passage through the complete digestive tract (Coghlan, 2002).

As mentioned before, one of the major fears among the public is that the transfer of genes will give rise to antibiotic resistance. This is a particular concern with regard to farm animals being fed with transgenic crops. The likelihood of a transgene being taken up by bacteria in the rumen gut seems small. Firstly, the transgenic DNA,

represents one gene among thousands of others in the plant genome, and must be cut from the plant genome through excision events occurring at random. The excised transgenic DNA must then survive the digestive enzymes from various tissues in the animal, such as the salivary glands, pancreas, etc. Most of the DNA will be rapidly digested into many small fragments. Naturally-competent rumen bacteria must then be able to take up the DNA, which is in itself a rare and random event. An antibiotic resistance gene, if it is still intact at this stage, must therefore compete with the rest of the DNA in the dietary sources for transfer into a bacterium. It is clear that the chances of an antibiotic resistance gene entering a rumen bacterium are extremely unlikely. However, although the risk is extremely low, it is not totally impossible. The consequences of such an acquisition must be considered during risk assessment and scientists and regulators are therefore considering alternative transformation technologies (Thomson, 2002).

Humans and animals consume significant amounts of DNA from a wide variety of sources, such as plants, animals, bacteria, fungi and viruses, on a daily basis. It has been estimated that a cow fed on GM maize, whether it be forage, silage or grain, will consume GM DNA at a ratio of 1:234000 to other DNA. This comprises only 0.00042% of the total dietary DNA intake (Beever and Kemp, 2000). Apart from the fact that ingested DNA is rapidly degraded, it appears that exposure to GM DNA will be negligible compared with exposure to normal unmodified crop DNA (Thomson, 2002).

2.2.4 THE FATE OF DNA IN THE SOIL

Another aspect of gene transfer is how the environment influences the transfer of genes. Soil can be seen as a potential reservoir of DNA molecules. The DNA in soil originates either from the lysis of dead plant, animal and microbial cells, or from the excretion of plasmid or chromosomal DNA by some microorganisms (Ogram *et al.*, 1987). It is possible that a fraction of extracellular DNA could escape the enzymatic activity of the DNases in the soil by the adsorption of the molecules onto sand or clay particles (Paget *et al.,* 1992), quartz, feldspar, heavy minerals and humic acids (Lorenz and Wackernagel, 1994). The adsorption capacity of clay minerals is very high. For instance, 1 g of pure montmorillonite can absorb up to 30 mg of DNA, which could represent 10^3 genomes of *E. coli* (Paget and Simonet, 1994). DNA can also resist nucleolytic attack by binding to soil minerals (Gallori *et al.*, 1994) and plant polysaccharides (Stewart and Carlson, 1986), thereby remaining available for the possible transformation of competent bacteria (Chamier *et al.*, 1993).

Bertolla and Simonet (1999) referred to studies that confirmed the survival of plant DNA in the soil. For instance, transgenic plant DNA could still be detected through PCR for longer than four months in laboratory experiments (Widmer *et al.*, 1996). Studies performed on field soil samples could detect specific sequences for more than two years after the specific crops had been harvested (Paget and Simonet, 1994; Gebhard and Smalla, 1999).

It is unclear whether gene transfer will be a notable phenomenon under natural conditions. From all the model studies, it is clear that the event of gene transfer will only take place at very low frequencies. In soil, there is a succession of extremely selective barriers to DNA being successfully transferred to a recipient organism. The recipient organism requires: (i) the release of DNA into the environment; (ii) the adsorption of DNA onto soil particles for protection against degrading enzymes; (iii) the presence of bacteria that are genetically adapted for natural transformation; (iv) proper conditions for the development of bacterial competence; (v) appropriate adsorption of DNA to the bacterial cell surface; (vi) the efficient uptake of DNA; (vii) chromosomal integration of the transforming DNA through recombination or autonomous replication; and (viii) gene expression by the recipient bacterium (Smith *et al.*, 1981). Furthermore, only a limited number of bacteria can act as recipients for transgenic sequences. These include those organisms that develop a stage of competence and ultimately exhibit tolerance to foreign DNA (Bertolla and Simonet, 1999).

2.2.5 THE OCCURRENCE OF GENE TRANSFER EVENTS

Gene transfer analysis has become important for biosafety reasons because of the pending release of GMOs into the environment. As a result, several studies were conducted to analyse gene transfer in the natural environment. The existence of so-called environmental hotspots for gene transfer was identified and these were shown to provide favourable conditions for horizontal gene transfer events to occur. These include the phytosphere, which consists of the phylloplane, phyllosphere, rhizoplane and rhizosphere. It is assumed that the plants in these spheres contribute to a microhabitat with a nutrient status that is favourable for genetic information to be transferred (Dröge *et al.*, 1998). The rhizosphere and rhizoplane contribute nutrients from root cells that are sloughed off as the roots grow into the soil, as well as through molecules escaping from the root cell wall (Wellington *et al.*, 1993). The phylloshpere and phylloplane contribute nutrients through frequent, natural conditions, e.g. nutrients being leached from leaves as a result of high humidity. Even the leaf surface may stimulate conjugation of DNA (Björklöf *et al.*, 1995).

Although it appears that biological and physical barriers strongly limit the occurrence of inter-kingdom gene transfer through natural transformation, the possibility of it happening, even under natural conditions, cannot be excluded (Bertolla and Simonet, 1999). Several studies have been undertaken to determine if transgenes from GM plants can potentially be transferred to microorganisms associated with the soil and plants. These studies were inclined to confirm that the event of DNA transfer would indeed be extremely rare (Gasson, 2000). For instance, in a study conducted by Schluter *et al.* (1995), no evidence of DNA transfer from plant to bacterium was found. However, *in vitro* studies provided quantitative data on the probability of such an event ever taking place. The probability was estimated at a maximum of 5.8×10^{-14} for an experiment in which 0.9 g of transgenic potato and

$6.4x10^8$ colony forming units (CFU)/ml bacteria were used. It is evident that the results obtained in this field of research could have an influence on the acceptance of all types of GM material in the future.

Gene transfer via conjugation could involve phylogenetically distant organisms, e.g. exchanges from bacteria to yeast and filamentous fungi. It has been shown that an *E. coli* yeast shuttle plasmid was transferred between *E. coli* and *S. cerevisiae* (Heinemann and Sprague, 1989; Nishikawa *et al.*, 1992), as well as between *E. coli* and *Schizosaccharomyces pombe* (Sikorski *et al.*, 1990). Plasmid transfer also occurred from *E. coli* into several yeast species, e.g. *Kluyveromyces lactis, Pichia angusta* (or *Hansenula polymorpha*) and *Pachysolen tannophilus* (Hayman and Bolen, 1993). A constructed plasmid containing T-DNA and compatible elements was transferred from *Agrobacterium tumefaciens* to *S. cerevisiae* (Bundock *et al.*, 1995). Dunn-Coleman and Wang (1998) even demonstrated that transfers between *Agrobacterium* and filamentous fungi were possible. Nielsen *et al.* (1998) stated that horizontal gene transfer was also likely to occur between protozoa (Schlimme *et al.*, 1997), nematodes (Adamo and Gealt, 1996), insect larvae and earthworms (Daane *et al.*, 1996).

How simple will it be for a transgene to interchange and be expressed between eukaryotic and prokaryotic organisms? Extracellular DNA can originate from a wide variety of prokaryotic and eukaryotic organisms. These two types of organisms differ considerably with regard to their DNA methylation processes, which could lead to differences in sensitivity towards the restriction and modification systems of competent bacteria. Another major difference is that, unlike eukaryotes, bacteria do not have mRNA excision and splicing mechanisms and are thus unable to translate intron-containing eukaryotic genes. Furthermore, their promoters have different structures with different consensus sequences. Consequently, for a eukaryotic transgene to be expressed in a bacterium, a prokaryotic promoter is required to be located upstream from the gene integration site. Another barrier to the transfer of genes between prokaryotes and eukaryotes is that the condensing effect of histones associated with eukaryotic DNA molecules could provide major interference in the uptake and recombination mechanisms in bacteria (Bertolla and Simonet, 1999).

The integration and expression of transgenes in microorganisms can be promoted by the presence of homologous sequences (Bertolla and Simonet, 1999). Importantly, in many case studies where bacteria were co-inoculated with transgenic plants, the recipient bacterial cells did not harbour any DNA sequences homologous to those bordering the transgenes. Should homologous sequences be present, it would help establish the transgene in the recipient and therefore also increase the probability of the detection of horizontal gene transfer events (Dröge *et al.*, 1998).

2.2.6 ASSESSING GENE TRANSFER EVENTS AND RISKS

The possibility of gene transfer from GM microorganisms into the environment or food, forms an important part of the safety evaluation for the release of GM

microorganisms. The consequences of gene transfer should be the main focus of such an evaluation, since it is clear that gene transfer is a significant event in bacterial evolution. Despite the available data on gene transfer, more emphasis should be put on the consequences of any possible transfers and especially the importance of a selective advantage that could be obtained by microorganisms. Unless the occurrence of a gene transfer event leads to a selective advantage in the recipient microorganism, the event in itself is unlikely to be of any great significance. However, a selective advantage could make even very rare genetic events something of great importance (Gasson, 2000).

Three different approaches are used to detect gene transfer events in nature. The first involves the detection of homologous genes by analysing the nucleotide or deduced amino acid sequence, which does not follow the general pattern of the evolutionary divergence of these organisms. This is often the only way of identifying gene transfer events across species boundaries. The second approach involves the investigation of gene transfer experiments under laboratory conditions. The third approach deals with more natural conditions, such as field studies and microcosms, and involves the analysis of lateral transmission of genetic information in these systems (Dröge *et al.*, 1998).

There are methods by which the industry makes an effort to curb the spread of transgenes in plants. The biotechnological giant, Monsanto, stated that it still avoids creating genetic manipulations in "risky" plants, such as sunflower and sorghum, since they can both freely hybridise with relatives that are already significant weeds. Furthermore, it tries to maintain a practice of not using GMOs in regions where plants have wild relatives and pursues approaches such as the notorious Terminator technology, which can cause host plants to produce sterile seed. Although biotechnological companies may find ways to reduce the likelihood of potentially troublesome transgenes escaping, it seems clear that there is still no substitute for a case-by-case risk assessment strategy (Holmes, 2002). Despite the evidence that exists for the overall improved environmental safety as a result of the wider use of recombinant DNA technology, it is not indicative that all GM products will be safe and therefore a case-by-case approach must be followed before commercialisation (IFT Expert Report on Biotechnology and Foods, 2000c).

In conclusion, although theoretical models and experimental studies show that the transfer of recombinant DNA from GM plants to plant-associated microorganisms occurs at extremely low frequencies, it is nevertheless an event that could happen. Therefore, questions relating to the implications of gene transfer events have to be addressed. It should be kept in mind that the issue of the frequency of occurrence is not that important, because even very rare events could have an ecological impact (Dröge *et al.*, 1998).

2.3 THE PUBLIC PERCEPTION OF BIOTECHNOLOGY

2.3.1 THE ISSUE OF TRUST

The history of biotechnology regulation in Europe and the USA is very different. In the USA, by the end of the 1980s, an active, often explosive, but relatively short public debate settled most of the main regulatory issues regarding genetic manipulation. Biotechnology was not believed to hold a special risk and regulation was exercised within already existing laws that addressed the known physical risks of new products. In contrast, the Europeans are engaged in a prolonged public debate that still has not ended in a feasible consensus. Regulators in Europe view biotechnology as a novel process requiring novel regulatory approaches (Thomson, 2002).

There appear to be major differences in the public's perception of food biotechnology in the USA and in Europe. A survey showed that significantly more Americans than Europeans answered questions such as the following correctly: (i) Do genetically modified tomatoes contain genes and normal tomatoes not? (ii) Can your genes become modified if you eat a genetically modified fruit? and (iii) Are genetically modified animals always bigger than normal ones? It is not surprising that Europeans approach food biotechnology with more suspicion (Gaskell *et al.*, 1999). The recent food safety scare as a result of bovine spongiform encephalopathy (BSE) has understandably made a large section of the European public more sensitive to the potential dangers inherent in industrial farming practices and the lack of effective oversight (Thomson, 2002).

The question of trust in regulation and regulatory bodies was investigated in Europe and the USA. Europeans voiced their level of confidence in being informed on the truth about GM crops by casting their votes for environmental (23%), consumer (16%) and farming (16%) organisations. National public bodies attracted only 4% of the votes, despite the fact that confidence was shown in the United Nations (UN) and the World Health Organisation (WHO). Americans regarded their regulatory authorities as far more trustworthy, with 90% of respondents supporting the United States Department of Agriculture (USDA) and 84% the Federal Drug Administration (FDA) (Gaskell *et al.*, 1999).

The Eurobarometer study, conducted between 1991 and 2002, was one of the most extensive surveys on biotechnology conducted in Europe. Four times during this period, 15000 people were interviewed to assess their attitude towards biotechnology. Except for a decline towards the end of the survey, people generally felt optimistic about biotechnology. The use of biotechnology for medical applications, e.g. the production of pharmaceuticals, vaccines and diagnostics, was accepted well. However, the field of food biotechnology was viewed with strong scepticism (Braun, 2002).

Bredahl (1999) conducted a study on consumer perception of two different GM products: yoghurt produced by a GM starter culture and a beer brewed with GM

yeast. The products differed in that the modified starter culture still remained in the yoghurt, while the modified yeast was filtered out of the beer. Despite the difference, the consumers' perception of it was not changed. Consumers still reacted with overwhelming fear of the unpleasant consequences of the consumption of GM products. This study also showed that, although the consumers understood the benefits of products made with GMOs, this did not compensate for their negative associations with these products. Grunert *et al.* (2001) found that consumers primarily associated conventionally produced products with safety and good health, while any kind of GM product was associated with poorer health and uncertainty.

A study by Baker and Burnham (2002) indicated that consumers who are not in favour of GMOs are best identified on the basis of what they believe, and not on the basis of who they are. Therefore, in the future it might be better for GMO producers to initially target early adopters who might be more willing to accept risks. Results also confirm that consumers are more accepting of GMO products with more tangible benefits for them (Lusk *et al.*, 2002).

A EU biosafety report published in 2001 that summarised 15 years of research on GM products and included 81 separate studies could find no evidence that these foods posed any new risks to the environment or human health (for a full-text review see http://europa.eu.int/comm/research/quality-of-life/gmo/index.html).

2.3.2 NATURAL IS BEST?

Another important factor that strongly influences people's perception of biotechnology and GMOs is that environmental issues have become part of the political mainstream. GMOs have successfully provided a means for environmental non-governmental organisations (NGOs) and Green parties to attract people to their causes. Organic farmers do not permit any GMOs in their products. Their success parallels the belief by many people that what is natural is simply better. However, agriculture is never a natural practice. Far less food would have been available in the world if chemical fertilisers did not exist. Many applications of genetically engineered crops have the possibility of improving the sustainability of farming, including organic farming, e.g. Bt cotton (Braun, 2002).

Although organic foods have their own niche market, they are not necessarily a better, safer or healthier option. The production of food the organic way could lead to food contamination by harmful bacteria (Avery, 1995). It should be comforting to know that never before have foodstuffs been subjected to such an extensive array of quality and safety checks as have foods derived from GM crops (Thomson, 2002).

2.3.3 THE ROLE OF ANTIBIOTIC RESISTANCE

Another legitimate concern among the public is the possible transfer of antibiotic marker genes from modified crop plants to pathogenic organisms. This concern has strongly contributed to the negative perception of genetic engineering as a technology that increases the problem of widespread antibiotic resistance. In safety

assessments and public debates, it is important to highlight that there are other environmental sources of the same genetic information that fall outside the jurisdiction of genetic engineering (Käppeli and Auberson, 1998). Genetic engineering alone cannot be held accountable for an increase in antibiotic resistance in the environment. It would be more responsible and realistic to acknowledge and question the widespread use of antibiotics in the animal farming industry (Witte, 1998; Feinman, 1998).

It is obvious that the public perception is largely shaped by the extensive debate on transgenic plants and GM foods. The literature is flooded with prominent and publicised debates focusing on the arena of GM plants. Environmental studies on transgenic plants would surely help improve the public perception of genetic engineering if it was emphasised that gene flow is not a risk but a natural process. It is only the nature of the transferred genetic information that will have an influence on the type of consequence that could occur (Käppeli and Auberson, 1998). A better understanding of concepts such as these could lead to more responsible and informed attitudes towards other kinds of GMOs.

2.3.4 ACTIONS REQUIRED TO PROVIDE PUBLIC WITH A BALANCED OPINION

One of the ways to improve the public's perception of genetic engineering is, for example, to emphasise that the process of deliberately altering plants genetically is not a new concept. What is new is an increased awareness of the biological hazards that are also present in traditional breeding. An improved scientific understanding of natural mechanisms such as gene transfer, gene acquisition and genetic variability has led to an increased alert (Käppeli and Auberson, 1998).

Thomson (2002) reported on an insightful speech given by the USA Agriculture Secretary, Dan Glickman, to the National Press Club in 1999. He laid out five important principles that should guide us in our approach to biotechnology in the future: (i) An arm's length regulation process: it is important that government regulators are involved with the companies that develop and promote biotechnological products and continue to protect public health, safety and environment; (ii) Consumer acceptance: this is based fundamentally on a sound regulatory process in which informative labelling could play a role; (iii) Fairness to farmers: the biotechnology industry must develop products that will give more options to farmers and show real, meaningful results; (iv) Corporate citizenship: the profit-driven biotechnology companies must respect the different roles of the arm's length regulator, farmer and consumer; (v) Free and open trade: organisations driven by unfounded, unjustified scientific claims cannot prevent trade in agriculture. A sound application of all of these principles could work together for a better perception of biotechnology by the public.

2.4 ETHICAL ISSUES IN BIOTECHNOLOGY

Regardless of whether environmental norms are products of human constructs or originate from the Divine, they place restrictions on freedom of action. These restrictions are imposed by humans as a result of the realisation that they are part of a community of interdependent parts, such as human generations and ecosystems. In western society at least, the relationship between humans and nature is the object of contradictory representations. On the one hand, we have nostalgia for a lost paradise, while a primitive fear of natural forces, as well as an urge to dominate nature, exist on the other hand. Technology cannot solve all human-made environmental problems; it is necessary that humans should also change their behaviour. Consequently, there is a need for codes of conduct that are informed by considerations of environmental impacts (Bourdeau, 2004).

According to Polkinghorne (2002), there are three current features that heighten public concern about the ethics of biotechnology. Increasing knowledge of genetics and the ability to perform genetic manipulations with genomes of plants and animals have resulted in much biotechnological development. This incredible power of intervention in nature raises the concern of whether this is an ethically questionable activity in itself. The integrity of nature is however, a complex issue, open to interpretation. In itself, the division between the natural and the unnatural cannot be of intrinsic ethical significance. For example, although heart transplants are just as unnatural as gene transplants, most people view them as being ethically acceptable. The pace of discovery in biotechnology is also great and has created the concern that a kind of technological compulsion will force developments ahead of thorough ethical consideration. Due to uncertainty about the long-term effects and environmental consequences of the genetic manipulation of organisms, there must be ethical limits on the use of what is technically possible. An example of the recognition of this is the moratorium on human germ-line therapy (Polkinghorne, 2002).

Biotechnology companies are considerably empowered by the technical experts they employ. This has raised public suspicion of the reliability and independence of this expert advice. In addition, transnational corporations are suspected of wanting to maximise profits by controlling the availability and dependability of their products. It is ethically correct that biotechnology is provided on an acceptable and just basis. However, this must neither deny reasonable reward for those who have invested enormous expense and risk in research and development, nor enslave small-scale consumers to large-scale suppliers (Polkinghorne, 2002).

There are those who equate the genetic manipulation of organisms with "playing God" and regard it as ethically unacceptable to interfere with nature in this way. Many religious people, however, would consider the responsible use of scientific skills as the exercise of God-given abilities. The ethical cannot simply be depicted as being equal to the natural, otherwise many of the developments in medicine could be viewed as morally wrong (Polkinghorne, 2002).

Every intervention in nature has the risk of unexpected outcomes. With regard to the environmental effect of GMOs, it is required from an ethical point of view that some form of precautionary principle is in place. This should not, however, result in total paralysis. Carefully controlled and monitored trials must be utilised to acquire the necessary knowledge on which ethically responsible decisions can be based (Polkinghorne, 2002). The precautionary principle will be discussed further in section 2.6.3.

Whenever there is a clash between two different ethically desirable goals, moral complications can emerge. There needs to be some acknowledgement of the common good if these types of problems are to be resolved. In particular, this includes a call for fairness in the policies of international corporations, as well as in the international regulation of trade in biotechnology. Naturally, the respect for safety must always be added as a universal ethical obligation. With regard to GM food safety, it does not seem to demand that the monitoring of techniques should differ from the assessment of unmodified foods (Polkinghorne, 2002).

The participation of at least three important parties will be needed in the search for sound decisions on ethical issues in biotechnology, namely the scientific experts, the general public and the community of people who will possibly benefit the most, e.g. certain farmers and sufferers from a particular disease. The experts will be able to assess possible risks and benefits of new developments and are obliged to perform this in as fair and balanced a manner as possible. However, final decisions cannot be made by them. They, as well as the community of beneficiaries, cannot be the judges in their own cause. The general public has an important influence on what is decided, but it is important that an informed public opinion is developed in order to make this influence more effective. There are some difficulties in this regard, since opposing single-issue pressure groups are continuously confronting each other in ethical debates. It is imperative that society establishes forums in which ethical issues can be discussed in a non-confrontational and truth-seeking way (Polkinghorne, 2002).

A great number of educational programmes will also be required in the future, since many people still lack the fundamental scientific understanding that is so important to draw well-informed, ethical conclusions. A good example is the case of irradiated food; although this is a very effective way of improving food safety, the public regarded this as an invisible hazard and refused to buy food labelled as such (Polkinghorne, 2002).

Bourdeau (2004) elaborated on a few fundamental questions asked by Naess (1973) regarding environmental ethics: (i) What are mankind's obligations towards the natural world? Our obligations demand qualitative and quantitative limits to our exploitation of nature; (ii) How are we to distribute the benefits and burdens that derive from respecting these obligations? The argument of international equity and justice comes into play, especially in the areas of the benefits of biotechnology and the property of genetic resources; (iii) What are the necessary policies and structures

to implement these obligations? Apart from the already existing national and EU policies and international conventions, there is probably a need for another worldwide body, e.g. a World Environment and Development Organisation (WEDO), that could counterbalance the World Trade Organisation (WTO).

According to the German philosopher, Hans Jonas, we must be guardians of nature and of future generations and our responsibility for the future lies in the fact that we are capable of compromising it. We are obligated to be careful to avoid undesired consequences and to gain knowledge through scientific research so that we will be able to assess the consequences of our actions (Bourdeau, 2004).

Thus far, moral and ethical concerns have not received as much attention as other issues in the GM debate, but it is quite possible that they will become more prominent in the future.

2.5 REGULATORY PRINCIPLES: NORTH AMERICA VS. EUROPE

There are important differences in the perception of the benefits and risks associated with genetic engineering in different countries. Some countries, like the United States, Canada, Argentina, Mexico and China, are adopting new GM crop varieties at a rapid rate (James, 2000). In general, citizens in these countries accept these new developments in biotechnology. Authorities in the USA regard GM varieties as not differing fundamentally from being substantially equivalent to their unmodified counterparts or conventional products. However, consumers in Western Europe and Japan hold a different view. Consumers in these countries are especially concerned with the issue of food safety and the environmental impact of GM crops. In the EU, regulators regard the conventional and GM varieties as being different because of the possible risks involved. As a result, the EU issued a moratorium in 1999 on the approval of additional GM crops for consumption (Nielsen *et al.*, 2003).

Whereas GM crops have become widespread in the USA since 1994, the commercial cultivation of these crops has not been embraced in Europe. Hails and Kinderlerer (2003) stated that, since 1998, the progress of GM crops through the regulatory system in Europe has almost come to a standstill. All regulations concerning the release of GMOs into the environment and the marketing of GM products have been thoroughly reviewed since then (Anonymous, 1998; Anonymous, 2001). This was caused by public concern about the potential consequences of the environmental release of some GMOs, as well as the desire for freedom of choice in the use of GM and non-GM products.

While GM foods have been introduced in the USA without any sign of consumer anxiety, consumers in the EU are becoming more anxious regarding the issue of GM food. One explanation for this could be that Europe now lives in a post-BSE age. In the wake of the epidemic, people believed that it has been brought on by unchecked, industry-driven alterations in farming practices and that government and the scientific authorities were wrong about their denials of any risk (Horton, 1999).

It seems that farmers and traders in the USA have a greater trust in science and view the scepticism of the EU regarding transgenic crops as invalid. This aroused the issue of hidden political agendas, in terms of which small scale and heavily subsidised European farming is protected. It is possible that GMOs could lead to a serious transatlantic trade dispute in the future, since the rules of the WTO are not designed to allow such scientifically unfounded discrimination (Braun, 2002).

The most tangible evidence of the difference in attitude of the Americans towards biotechnology was the appearance of biotechnological companies on Wall Street. Although Europe also has many biotechnological companies, the GM debate on this continent has been more intense than in the USA since GMOs first became prominent in the 1970s (Thomson, 2002). In essence, the American regulatory system for transgenic crops and food produced from them is largely *product* based, whereas the regulation system in Europe is *process* based (Miller, 2001). In other words, the USA only evaluates the measurable properties of a crop or product, whereas the Europeans are interested in whether a crop is bred through genetic engineering or conventional techniques. In comparison to crops produced through methods such as irradiation, mutations, hybridisation and selection, biotechnologically-derived crops are viewed in a much more stringent light by the European regulatory system (Braun, 2002).

Regarding the controversial issue of labelling, the USA and Canada require labelling only when the allergenic or nutritional composition of a product has been changed through genetic engineering. In any other instances, the labelling of food containing GM ingredients is totally voluntary (Nielsen *et al.*, 2003) and has been implemented to establish markets for certain food niches that are desired by consumers (IFT Expert Report on Biotechnology and Foods, 2000b). The EU has taken a more extreme stance and has made the labelling of all food and food ingredients containing genetically engineered DNA or proteins above a 1% threshold as mandatory (Nielsen *et al.*, 2003). In general, the USA regards the new labelling system as unworkable and very expensive for producers (Braun, 2002).

In Europe, primary legislation concerning the regulation of the deliberate release of GMOs is drawn up by the European Community. Domestic legislation then implements this in the individual member states. In the UK, a framework of committees exists that is involved in the regulation of and providing advice on the safety of humans and the environment. There are no GM-specific laws in the USA and, depending on the nature of the product, one of three different agencies can review a specific product, namely the USDA, the Environment Protection Agency (EPA) and the FDA (Hails and Kinderlerer, 2003). These federal agencies also support a programme of peer-reviewed research grants that focus on current and future issues of safety to increase the existing knowledge regarding crops and food derived from biotechnology (Thomson, 2002).

Europe is now in a position to restart the regulatory process (Hails and Kinderlerer (2003). In a recent report the EU recognised the potential benefits of

biotechnology and the need for the realisation of these benefits for Europe (Anonymous, 2002). In response, the UK organised a national dialogue on genetic modification in 2003. European countries were also required to implement a new directive, the European Union Directive on Deliberate Release. This directive acknowledges the importance of issues such as respect for ethical principles and public involvement in proposed releases of GMOs. The well-known Cartagena Protocol also came into force in September 2003. This protocol is an international trade/environment treaty secondary to the Convention of Biological Diversity (CBD). It requires countries to involve the public in decisions concerning GMOs (Hails and Kinderlerer, 2003). The Cartagena Protocol will be discussed further in the next section.

Europe will have to seriously consider decisions on the commercialisation of GM crops and products soon. With support from Canada and Argentina, in 2003 the USA initiated a dispute with the EU in relation to its effective moratorium on GM products. The argument related to the unfair trade restrictions that have been placed on agricultural and food products from the USA (Hails and Kinderlerer, 2003).

In other countries, the regulatory systems are usually intermediate versions of these two models. A range of triggers are used to bring products into the regulatory system, e.g. Europe uses transgenic technology, while Canada uses the concept of novelty as a trigger (Hails and Kinderlerer, 2003). In South Africa, the Department of National Health and Population Development regulates existing laws that govern the import of GMOs (Plant Pest Act) and novel foods and ingredients. In 1998, as a new GMO Act came into effect, an advisory scientific committee replaced the long-standing biosafety committee, The South African Committee on Genetic Experimentation (SAGENE). Under the auspices of the Ministry of Agriculture, all environmental and food safety assessments regarding GMOs will be technically reviewed by the advisory committee. The labelling policy in South Africa will probably adopt the *Codex Alimentarius* guidelines (Watson, 1998). The *Codex Alimentarius* is an international food standards body created in 1961 and an agency of the WHO and the Food and Agriculture Organisation (Mitten *et al.*, 1999).

2.6 RISK MANAGEMENT

2.6.1 AIM AND GENERAL CONSIDERATIONS

Risk assessment is already a well-established feature of biotechnology. Over the years, safety practices for handling microorganisms have evolved from working with human diseases. These safety practices were formulated into practical and effective rules for containment on the basis of fundamental knowledge about the causal agents. Similar rules have been developed and are being employed for the release of organisms into the environment (The Safety in Biotechnology Working Party of the European Federation of Biotechnology, 1999).

The aim of risk assessment is to target and evaluate any potential negative effects that GMOs could have. Although these effects can be direct, indirect, immediate or future, the cumulative and long-term effects on the environment and human health must be considered. In short, the risk assessment procedure for any given food product, for example, looks specifically at how a GM product was developed. It also examines any risks associated with new, introduced gene products (e.g. allergenic proteins) within the modified food product and with possible gene transfer. The identification of any characteristics that might cause negative effects, the evaluation of their potential consequences and the possibility of their occurrence, determine the overall risk of a GMO. After a risk has then been estimated, the application of management strategies must be implemented (Renault, 2002).

The rules currently being followed in risk assessment are the best practicable ones. They address the classes of known and ascertainable risks obtained from experience and the reasonable conclusions drawn from experience. However, these rules cannot offer absolute guarantees on safety, because they operate on the basis of available knowledge and therefore there is some degree of uncertainty. The parties involved are obligated to be up to date and as complete in their knowledge as possible.

There is also an overwhelming urge to innovate towards sustainability. In light of the ongoing uncertainty about, and unpredictability of the long-term effects, there is also an obligation to monitor and record at all levels. These levels range from the individual managers' responsibility to a more general societal obligation to keep using freedom of expression and the channels for criticism (The Safety in Biotechnology Working Party of the European Federation of Biotechnology, 1999).

Several risk assessment schemes relating to field releases are currently in use in the USA, EU and other countries that form part of the Organisation for Economic Cooperation and Development (OECD). The Safety in Biotechnology Working Party of the European Federation of Biotechnology (1999) listed a number of consensus documents prepared by the OECD on monitoring conditions for the release of organisms into the environment (OECD, 1992; 1994a; 1994b; 1997). Most of the OECD documents are also available online (http://binas.unido.org/binas/library.html).

Of all the field trials in the OECD's Biotrack database in October 1998 (http://www.olis.oecd.org/biotrack.nsf; the database has only been updated until 1999), the proportions of projects involving bacteria, viruses and fungi are listed as ~1%, ~0.3% and ~0.2%, respectively. The number of projects involving the possible release of microorganisms is small in comparison to those involving plants. The OECD and the United Nations Industrial Development Organisation (UNIDO) are responsible for the Biotrack database, which provides records of field trials of GMOs that have taken place in OECD member countries, as well as data from other countries supplied by UNIDO's Biotechnology Information and Advisory Service. The UK also maintains a database (http://www.shef.ac.uk/uni/projects/doe/register.html)

of applications received by the Secretary of State for the release of GMOs into the environment (The Safety in Biotechnology Working Party of the European Federation of Biotechnology, 1999).

The legislation and regulations regarding release of GMOs are broadly similar in most countries. Guidelines require several obvious guarantees that have to be met for the approval and release of GMOs and their derived products. These guarantees usually include a thorough definition of the introduced DNA sequence and the elimination of any sequences that are dispensable in the expression of the desired property; the absence of any selective advantage that could allow the transgenic organism to become dominant in natural habitats; no danger to human health and the environment due to the transformed DNA; as well as a definite advantage to both producers and consumers (Pretorius, 2000).

2.6.2 THE PROCESS OF RISK ASSESSMENT

The Safety in Biotechnology Working Party of the European Federation of Biotechnology (1999) stated a few general definitions for four levels of concern or risk classes of microorganisms, which are based on scenarios of increasing severity (see Table 2.1). The assignment of a microorganism to an appropriate risk class is based on its properties, regardless of the technique by which it was constructed, and by then scoring it against a set of values that need to be protected. These values can include human, animal or plant health, biodiversity, as well as agronomic values. The highest score for any one value will determine the proper risk class. In some cases, the available knowledge will be reliable, while in other cases appropriate experimental tests (first in contained conditions and then small-scale field trials) will

Table 2.1 General risk classes of microorganisms for environmental application.

Risk Class	Adverse effects	Other remarks
1	None or highly unlikely.	Organisms are considered to be safe.
2	Possible, but unlikely to represent a serious hazard. Local effects possible which can recur spontaneously or be controlled by available treatment or preventive measures.	Spread beyond the area of application highly unlikely.
3	Serious local effects are likely.	Spread beyond the area of application unlikely. Available treatment and/or preventive measures.
4	Serious effects to be expected locally and outside area of application.	No treatment and/or preventive measures available. Out of question for any environmental use.

be necessary for environmental risk assessment on the basis of measurable properties, such as competition, horizontal gene transfer and the potential for monitoring and control.

Depending on the type of GMO, its intended use and the receiving environment, the required information for risk assessment may differ in nature and detail. The risk assessment process may give rise to a need for further information about certain aspects, while information about other aspects may not be relevant in some instances. Table 2.2 contains a description of the different aspects that can be involved in a risk assessment process. The risks associated with GMOs or their related products containing detectable amounts of replicable genetic material should be considered in the context of the risks imposed by the unmodified recipient or parental strain in the receiving environment (Jank and Gaugitsch, 2001).

A schematic approach to data generation and the subsequent risk assessment of GMOs and GM products is shown in Fig. 2.1.

Table 2.2 The different aspects of risk assessment, depending on the case. Adapted from Jank and Gaugitsch (2001).

• Recipient organism or Parental organisms	Their biological features, which can include taxonomic status, common name, origin, centre of origin and genetic diversity (if known) and description of habitat where organism may proliferate.
• Donor organism(s)	Taxonomic status, common name, source and relevant biological features.
• Vector	Characteristics including identity (if any), source/origin and its host range.
• Insert(s) and/or characteristics of modification	Genetic characteristics of inserted DNA with its specified function and/or characteristics of the introduced modification.
• GMO	Identity of GMO; differences between the biological characteristics of the GMO and the recipient or parental organism(s).
• Detection and identification of the GMO	Proposed detection and identification methods, including their specificity, sensitivity and reliability.
• Information relating to the intended use	Information relating to the GMO's intended use, including the difference in the use compared to the recipient or parental organism(s).
• Receiving environment	Information on the location, geographical, climatic and ecological characteristics and relevant information on biological diversity and centres of origin of the receiving environment.

2.6.3 THE PRECAUTIONARY PRINCIPLE

In order to maximise the benefits and minimise the risks involved in any new technology, it is imperative that control is exercised through a set of guidelines or regulations (Braun, 2002). Appropriate risk assessment is important for the establishment of food legislation, as well as for trade issues. Scientific knowledge and input is an important prerequisite for a suitable risk assessment process. The science behind the concept of food safety and of risk management based on risk assessment is not fully understood by consumers and politicians, and therefore it

often happens that politicians hastily enforce action and define legislation in the light of certain events without any sufficient scientific data. All this has led to an important issue in the modern approach to food safety management, namely the "Precautionary Principle" (Anklam and Battaglia, 2001).

This principle is used in situations of scientific uncertainty and where no scientific proof exists that a certain product or organism will not cause serious harm. According to Pascal (2000), the sound application of this principle does not mean the abandonment of science, but rather calls for more science. Until scientific uncertainties become certainties, decision makers must consider this as a basis for taking action. Concerning the implementation of this principle, it is important that a lack of scientific knowledge or consensus should not necessarily be interpreted as an indication of a particular level or absence of an acceptable risk.

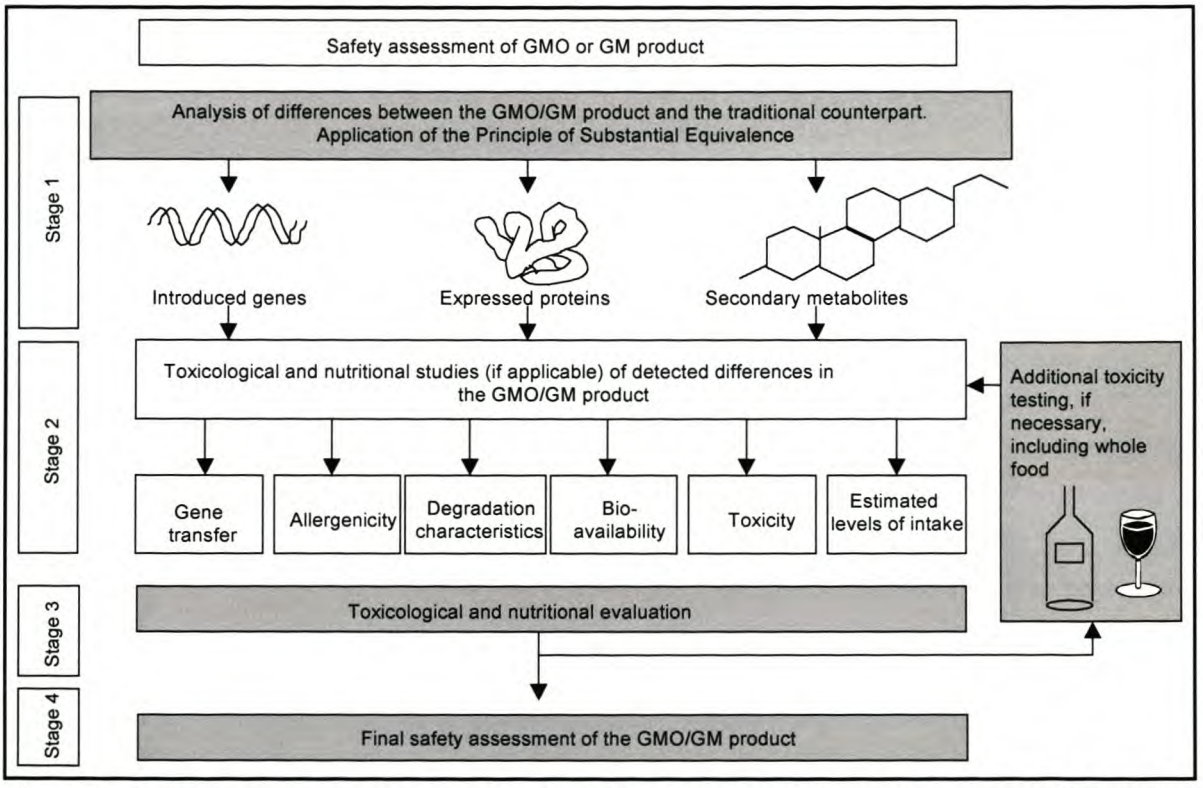

Fig. 2.1 Safety assessment strategies for GMOs and possible subsequent GMO-derived foods. Adapted from Kok and Kuiper (2003).

2.6.4 THE CONCEPT OF SUBSTANTIAL EQUIVALENCE

After the first initiatives regarding the development of strategies for the safety assessment of foods and feed derived from GMOs, a concept of comparison was established. This concept is based on comparisons with the traditionally bred or natural counterpart, which obviously has a history of safe use. The OECD elaborated this concept further and coined it the "Principle of Substantial Equivalence" (OECD, 1993). The aim of this principle was to establish a scientifically sound approach that would also meet with global acceptance. However, controversy followed this principle when it became clear that it left much space for individual

interpretations. This hampered the safety assessment process and it was established that the principle could only be applied on the basis of a thorough compositional analysis of both the GM variety under scrutiny and its traditional counterpart. If any differences in composition were identified, targeted toxicological and nutritional studies should be conducted to assess the safety for humans. The Principle of Substantial Equivalence represents a mere tool to identify potential differences and forms part of a comprehensive comparative safety assessment approach. Since the "Substantial" part of the Principle has led to some misinterpretations, Kok and Kuiper (2003) suggested that the Principle should be renamed the "Comparative Safety Assessment" approach. The comparative nature of the safety assessment is better outlined by this phrase and also avoids the idea of it being a safety assessment in itself.

The concept of substantial equivalence is thus a mere practical approach to the safety assessment of GM foods. Since it is not a safety assessment in itself, it is not designed to characterise hazard. It is only used to structure the assessment of the safety of GM food relative to the conventional counterpart. An objective safety assessment can only be made after all agronomic, genetic and chemical aspects have been taken into account. The nature of these differences and whether or not they are well characterised, determine the type and extent of any further studies (Thomson, 2002).

When assessing the risk of microbial strains that have been genetically modified, Renault (2002) further suggested that the issues of safety of the new gene product and gene transfer should be examined on the widely recognised case-by-case basis. Whether the new gene(s) will create undesirable functions in the new host could be examined by a strategy similar to an EU-founded program named "Express-Fingerprints". This system specifically tests the potential side effects of genetically driven mutagenesis compared to conventionally-induced random mutagenesis. Although genetically-driven mutagenesis is considered to allow targeted modifications and thus avoid additional mutations, the formal demonstration of this technique's directness has not been implemented, due to the fact that, until recently, side effects could only be determined with difficulty. In the "Express-Fingerprints" program, mutants of *Lactococcus lactis* obtained through chemical mutagenesis and gene technology were compared analytically by 2D gel electrophoresis and DNA microarrays. The relevant changes in the expression levels of at least 450 cytoplasmic proteins and the transcription of more than 2 100 genes larger than 89 base pairs, was considered. This program identifies possible side effects, and points out deregulation due to the wanted change itself. In the end, it can determine if changes in addition to the expected ones occurred, thereby increasing knowledge of gene function.

If the construction technology itself is not considered to influence safety, another important issue should be acknowledged. If it could be reasonably accepted that many mutants are substantially equivalent to naturally occurring strains, which extra

genes could be added without giving an organism the GMO stamp? GMOs are often constructed from a pool of genes present in the same or a less closely related species and it is now evident that many similar transfers occur in nature (Renault, 2002). In this regard, Renault (2002) mentioned internal transfers in bacteria, e.g. gene shuffling occurring on chromosomes (Delorme *et al.*, 1994; Guedon *et al.*, 1995; Schouler *et al.*, 1998), plasmids (Nardi *et al.*, 1997; O'Sullivan *et al.*, 2000), transposons (Immonen *et al.*, 1998) and phage-related elements (Durmaz and Klaenhammer, 2000; Brondsted *et al.*, 2001). All this confirms that the establishment of the limits of the "natural gene pool" is not a simple task.

2.6.5 THE ISSUE OF LABELLING AS A PERCEPTION OF RISK

Despite the fact that ample scientific evidence exists that current GM food poses no new risks, many civil society groups and a huge proportion of consumers are still not convinced. There is a belief that the mandatory labelling of GM foods will empower consumers in selecting their own diet and enhance the long-term monitoring of GM foods for the detection of any unanticipated risks (Smyth and Phillips, 2003).

An estimation of the sales impact of a few labelled products indicated that, despite hostility toward GMOs, sales did not decrease when the label revealed the product to contain GMOs (Eurobarometer, 2000; Noussair *et al.,* 2001). There was a huge difference between opinions and behaviours regarding products containing GMOs. Noussair *et al.* (2002) asked whether the hostility of the "citizen" becomes attenuated when it is placed in the role of a "consumer". An economic investigation by them revealed that this was not necessarily the case. This consumer reaction was probably the result of most customers not noticing the labelling and thus not realising that the purchased product contained GMOs. Labels that are not looked for are simply not noticed. Their research further showed that, for a specific product, the average consumer's willingness to buy the product declined by ~30% as soon as it was realised that the product contained GMOs. It was suggested that a standardised logo might be a method of clearly transmitting information.

In retrospect, it is a mistake to link labelling to safety. Additives which have been well examined and proved to be safe have gained a negative image. In the process, it has prevented informative labelling about the use of gene technology, which might only have contained factual information without any threats regarding safety aspects. Labelling should ideally be a means of providing information and not a safety warning (Mahler, 1999).

2.6.6 THE CARTAGENA PROTOCOL ON BIOSAFETY

Although there are many organisations around the world that are involved in the regulation of GMOs, the focus in this overview will be only on one of these. A Convention known as the Cartagena Protocol on Biosafety (CPB) (http://www.biodiv.org/biosafety) plays an important international role and seeks to protect biological diversity from the potential risks imposed by GMOs. The specific

objectives of this Protocol are to make a contribution to ensuring an adequate level of protection in the safe transfer, handling and use of GMOs that may have negative effects on the conservation and sustainable use of biological diversity, as well as on human health. The specific focus is on transboundary movements and the establishment of a so-called Biosafety Clearing House (BCH). The BCH assists countries in the implementation of the Protocol and facilitates the exchange of information. This Protocol has been called a breakthrough in that it supports the "precautionary approach" as a principle of international law and places the environment next to trade-related issues internationally (Jank and Gaugitsch, 2001).

In 2000, more than 130 governments signed the Cartagena Protocol agreement on the transboundary transport of living GMOs. Importing countries concerned about the safety of GMOs were given the right (on the basis of the precautionary principle) to prevent imports, even without scientific evidence that the GMOs would be harmful (Thomson, 2002). The proposals were limited to GMOs that might have adverse effects on biodiversity and did not cover the non-viable products of GMOs, e.g. processed foods and feeds, health care and pharmaceuticals products, products intended for contained use, such as in research and manufacturing, and commodities not intended for deliberate release into the environment, e.g. soybeans used for food processing (Gibbs, 2000).

2.6.7 FUTURE ISSUES IN RISK MANAGEMENT

For the future management of risks in the food industry, there is a strong need for worldwide networking between food control laboratories, reliable data banks and global monitoring studies. Of significant importance is also the need for the proper education of risk assessors and managers (Anklam and Battaglia, 2001). Decisions involving GMOs will require work and the cooperation of parties involved in areas of risk assessment, socio-economic considerations, and public awareness and participation (Jank and Gaugitsch, 2001).

The long-term development of international trade and regulation will certainly be influenced by cross-cultural differences in acceptance. The question is how the European precautionary regulation will influence European confidence in products derived from environments with less precautionary approaches to regulation. A worthy case for research is the long-term impact that the international diversity of public acceptance of food technologies could have on the ongoing development of world trade, as well as on political structures and international science policy. It may also be worth researching whether the potential for reduced environmental impact will increase the acceptance of genetically modified foods (Da Costa *et al.*, 2000).

The scientific tools of functional genomics (Kuipers, 1999) and DNA microarrays (Kuipers *et al.*, 2000) can be further exploited to develop concrete risk assessment procedures for GMOs used in food production. Numerous safety assessments of GM crops and foods have already been carried out. Thomson (2002) mentioned a few respected organisations that are in agreement that there is no evidence that

suggests that the GM foods on the market today are unsafe and that GM plants pose greater health or environmental risks than those modified by conventional breeding methods. These organisations include the National Academy of Science in the USA (http://www.nas.edu), the United States Congressional Committee on Science (http://www.house.gov/science), the Royal Society of the UK (http://www.royalsoc.ac.za), the Nuffield Foundation (http://www.nuffield.org), the European Molecular Biology Organisation (http://www.embo.org), as well as the Royal Society of South Africa (http://www.uct.ac.za/org/RSSA).

According to an expert report on biotechnology and foods, biotechnology-derived foods do not necessitate more stringent safety standards than those that are used for conventional foods. The greater precision by which genetically modified foods are produced can lead to foods that are better characterised and more predictable. Consequently, this could lead to a more reliable safety assessment process (IFT Expert Report on Biotechnology and Foods, 2000a).

In the end, the consumer should be educated and empowered to make well-informed decisions following proper risk assessment and a clear demonstration of safety (Viviers and Pretorius, 2002). Research should then be totally unbiased.

2.7 ROLE/RESPONSIBILITY OF THE SCIENTIFIC COMMUNITY

Both scientists and consumers have a significant role and shared responsibility in the debate on GM food safety and quality (Anklam and Battaglia, 2001). Apart from the need for information to flow from scientists to the public, it is equally important that scientists and government understand the issues and concerns of the public. Methods must be in place through which public views can be fed back into the decision-making process. As mentioned before, the UK made an effort in 2003 to promote open dialogue on genetic modification. This culminated in an organised national dialogue, carried out by an independent steering board, in which the public had the opportunity to question scientific experts (Hails and Kinderlerer, 2003).

Hails and Kinderlerer (2003) referred to an interesting report, "Science and the Public" (The Office of Science and Technology and the Wellcome Trust, 2002), that concluded that many British people were really enthusiastic about science and its benefits. However, what is important for trust is the perceived independence of any source of scientific information. It was found that university scientists, along with those working for charities, documentaries and television news, were high on the trustworthy list. Further down the list were environmental groups, well-known scientists and the popular scientific press. Politicians and newspapers were found at the bottom of the list. The report stated that confidence in the regulatory system and the government is low.

In another report, Hails and Kinderlerer (2003) investigated the attitudes of scientists (Wellcome Trust, 2001). The scientists in this survey believed it to be their responsibility to communicate their science and were keen on doing so. However,

the media was perceived as a barrier to better dialogue. Many believed that the media coverage of GM foods had confused the public's understanding of certain issues and that it made them more careful about scientific issues in general.

While the public ranks newspapers very low on their trust list, the scientific community is also concerned about GM reporting in the media (Hails and Kinderlerer, 2003). Researchers should build a relationship of mutual trust with journalists, since they are the key means of carrying the dialogue to the general public (Braun, 2002). Such a mutual understanding could help eliminate misunderstanding and determine the significance of new research.

While scientists are pulled into the political arena by both the proponents and opponents of gene technology, it is imperative that scientists remain honest and conscientious. They should stand apart from political considerations and not become cynical manipulators of public opinion. The scientific agenda must be determined by societal concerns, but the evaluation process must remain scientifically based (Mahler, 1999). Specialist input from researchers at the cutting edge of science to key decision makers could lead to the development of more realistic policies in the future.

One important feature of the GM debate is the seeming disagreement between scientists. Both opponents and proponents claim to have definite scientific evidence to support their cases. Two existing mechanisms could give guidance in the case of scientific disagreement. One of them is the highly acclaimed process of peer review, according to which experts in the same field criticise scientific research anonymously. Failure to publish in peer-reviewed journals means that scientific research has failed the test of strict and independent criticism. The voice of respected scientific institutions, such as the Royal Society, for example, forms the other mechanism. The reports that such societies produce should be treated with the authority that reflects the evidence behind it (Hails and Kinderlerer, 2003).

2.8 THE DETECTION OF MICROORGANISMS IN THE ENVIRONMENT

The major goal of ecological studies in microbiology is to understand the life of specific bacteria in their habitats and their interaction with their environment and other biota. Since the introduction of recombinant DNA technology and improved immunological methods, the diversity of methods available for tracing microorganisms in the environment has increased considerably (Schloter *et al.*, 1995).

The classic approach to characterising the presence and abundance of specific microorganisms has been the use of selective growth conditions. Unfortunately, many microorganisms cannot be cultured under routine laboratory conditions (Baek and Kenerley, 1998). Brockman (1995) reported that only 0.01-12% of the soil and aquatic microbial communities are commonly cultured. Culturing techniques only identify those viable species that are easily cultured *in vitro* and fail to isolate

organisms that grow poorly in culture or have particular growth requirements (Baker *et al.*, 2003). Under adverse environmental conditions, bacteria are capable of developing a survival strategy known as the viable but non-culturable state (VNC) (Oliver, 1993). Physicochemical factors such as nutrient concentration, temperature, aeration, etc. can induce entry into the VNC state. The influence of these parameters on bacterial species can be highly variable (Lleó *et al.*, 1999). Cells in the VNC state are alive and metabolically active, but are unable to form colonies on plates (Barer *et al.*, 1998). When optimal environmental conditions are restored, VNC cells can again resume active growth (Whitesides and Oliver, 1997; Lleó *et al.,* 1998). Standard methods of microbial detection (e.g. plate counts) may therefore not always be adequate, because they are unable to detect microorganisms in the non-culturable state (Lleó *et al.*, 1999). Techniques capable of detecting non-culturable cells (e.g. PCR, fluorescent monoclonal antibodies, specific DNA probes, etc.) allow the direct and sensitive detection and quantification of specific genes and the metabolic ability of microorganisms (Baek and Kenerley, 1998) and have been successfully applied in the detection of VNC bacteria in different environments (Bej *et al.*, 1990; Huq *et al.*, 1990; Islam *et al.*, 1993).

PCR is a very attractive tool for the detection of specific microorganisms in studies of microbial ecology and environmental microbiology (Steffan and Atlas, 1991). The considerable ease and rapidity of the application of PCR includes analysis without the necessity for culturing microorganisms from samples (Brockman, 1995). Primers can be designed for the amplification of DNA sequences specific to particular taxonomic groups, and will allow the detection of viable, non-viable and dormant cells (Baker *et al.*, 2003). However, the quantification of a specific target population can be weighed down by the fact that minute differences in any of the parameters that affect the efficiency of amplification can drastically affect the outcome of the reaction products (Baek and Kenerley, 1998). Therefore, techniques such as quantitative competitive PCR (QC-PCR) have been developed, in which target DNA is titrated by co-amplifying the target DNA with known quantities of an internal standard (Gilliland *et al.*, 1990). By using QC-PCR, quantifiation is independent of cycle number or the concentration of primers or deoxynucleotide triphosphates (Steffan and Atlas, 1991; Leser, 1995). PCR is a particularly suitable method for analysing environmental samples, for it has been demonstrated that the technique can be used to detect low numbers of a specific bacterium against a large background of organic material and other prokaryotic and eukaryotic cells (Tsai and Olson, 1992; Tiem *et al.*, 1994). For example, it was possible to demonstrate the presence of non-culturable forms of *Enterococcus faecalis* in lake water and to quantify their DNA and the corresponding concentration of non-culturable cells (Lleó *et al.*, 1999).

Baker *et al.* (2003) suggested that three important criteria should be met when PCR is to be used reliably for the detection of non-indigenous organisms in pristine environments: (i) the choice of appropriate primers; (ii) the establishment of the

primer specificity through widespread laboratory trials; and (iii) the careful and stringent selection of positive and negative controls. It is imperative that the specificity of chosen primers is confirmed experimentally. Primers should be tested against type cultures of the target species and environmental samples seeded with the target species in positive controls. They should also be tested against extracts of pristine samples in negative controls before they are used in the analysis of environmental samples.

Antibody-based detection allows the sensitive quantification and visualisation of cells *in situ*. Depending on the specific technique used, all cells, whether culturable or not, carrying a recognisable antigen can be traced. Modern biophysical techniques such as flow cytometry, confocal laser scanning microscopy, image analysis and luminometry, together with monoclonal antibodies, make it possible to trace microbes in the environment (Schloter *et al.*, 1995).

Recent advances in the use of fluorescent stains in flow cytometry (FCM) have greatly increased the rapid detection and viability measurements of microbes in homogenous and heterogeneous microbial populations. FCM has found many applications, e.g. to enumerate, differentiate and identify microorganisms, to determine cell protein and DNA content, to analyse the physiological state of individual cells and the interaction of antibiotics, drugs and antimicrobials on microbial cells (Noble-Wang *et al.*, 2004).

The presence of one or more unique markers is important to identify inoculant organisms against the natural background of indigenous organisms in environmental samples (Prosser, 1994; Jansson, 1995). The detection methods based on cultivation, immunology, flow cytometry or the estimation of nucleic acid or other cellular macromolecules utilise selected markers in such a way that fits the requirements of the method as well as the markers (Van Elsas *et al.*, 1998). Van Elsas *et al.* (1998) listed a few popular marker systems, which can be either intrinsic to the host cell, extraneous or relate to a genetic modification performed. The presence of transgenic DNA in GMOs provides great possibilities for their detection. Many bacterial, fungal and viral gene sequences of interest are available to use for the detection of specific organisms or their genes in the environment. By means of the careful comparison of candidate sequences with those in databases, specific primers and probes can be designed for these genes or sequences (Van Elsas *et al.*, 1998).

Unique and easily identifiable markers are required for monitoring of GMOs in the environment (Errampalli *et al.*, 1999). The use of molecular markers for detection of GMOs in the environment has been researched extensively (see Greer *et al.*, 1993; Akkermans *et al.*, 1994, Prosser, 1994 for reviews). The importance of DNA probes and markers in studying the ecology of GMOs and wild-type microorganisms that are non-culturable is discussed by Akkermans *et al.* (1994). Prosser (1994) elaborated on the molecular marker systems used for the detection of GMOs in the environment,

such as antibiotic resistance, *lac*ZY (β-galactosidase), *xyl*E (catechol 2,3-dioxygenase), *tfd*A (2,4-dichlorophenoxyacetate) and *lux* (luciferase).

Green fluorescent protein (GFP) became available as a novel marker in 1994 (Chalfie, 1994). It is more stable than plasmid-borne genes when introduced into the bacterial chromosome and therefore also minimises the possible transfer of marker DNA to indigenous microorganisms. This marker system has gained wide use in environmental applications since 1996, e.g. the study of the dynamics and distribution of *gfp*-labelled bacteria in soils, water, rhizospheres, activated-sludge, biofilms and root nodules, as well as the study of gene transfer between bacteria in biofilms and on phylloplanes (Errampalli *et al.*, 1999).

Microorganisms undergo a variety of processes after their introduction into the environment, including growth, death, physiological adjustment, conversion to non-culturable cells, physical spread and gene transfer. Using a single method for detection and risk evaluation is likely to provide a potentially biased and partial view of the ecology of the organisms (Van Elsas *et al.*, 1998). Selective plating methods will only determine the culturable part of inoculant organisms and will not detect VNC cells. Direct microscopic methods (e.g. specifically immunofluorescence) will provide information on the total inoculant cells present, without determining their viability or culturability (Postma *et al.*, 1988). Several staining techniques (Hobbie *et al.*, 1977; Kepner and Pratt, 1994; Korber *et al.*, 1996; Boulos *et al.*, 1999; Hope and Wilson, 2003) and other methods, e.g. the direct viable count (Heijnen *et al.,* 1996) can be used to assess cell viability. In order to compile precise information about the fate of a specific microorganism in the environment, it is therefore often necessary to combine two or more detection methods. In the combination of methods, greater certainty regarding the clear detection of specific target strains and the dynamics of different parts of populations can also be obtained (Schloter *et al.*, 1995).

The nutrient and moisture status of soil strongly affect the physiologies of microbial cells. In turn, this can have an effect on their detectability (Roszak and Colwell, 1987; Van Overbeek *et al.*, 1997). The nutrient-limited nature of soil potentially has an influence on the detectability of microbes by means of plating or any other cultivation-based method. Most soils are very heterogeneous, which presents particular problems for environmental monitoring, e.g. the difficulty in obtaining representative sampling (Van Elsas and Smalla, 1996). The difficulties encountered with soil act as an example of the problems faced when using environmental detection methods. Other open environments, e.g. aquatic systems and sediments, most likely present similar problems for microbial detection (Van Elsas *et al.*, 1998).

2.9 THE DETECTION OF GMOs IN FOOD

The available methods for the detection of GMOs in food were recently reviewed by Ahmed (2002). The most commonly used methods include Southern blotting and

PCR analysis for the detection of transgenic DNA and immunological tests for the detection of GM proteins (Table 2.3). However, all of these methods require detailed knowledge of the specific GMO to be monitored, in particular regarding the specific sequences used for the modification.

PCR analysis is the most sensitive method, even more so than conventional protein detection tests. Specifically, there are a few categories of target sequences in transgenic DNA that are being used for PCR-based detection. The first category involves those sequences that regulate the expression of an inserted gene. The second category refers to those genes that are used as genetic markers, e.g. antibiotic resistance genes, and the third category includes the target genes that are expected to be used in certain industries, e.g. those genes that will be advantageous in the wine industry when expressed in a wine-making yeast (Gachet *et al.*, 1999). The PCR technique can therefore easily detect GMOs by targeting genes that are introduced into the modified organism because of primers that amplify sequences from the cloned genes or from the sequences flanking these genes.

There are still unresolved issues in the methods for detecting the use of GMOs. Certain elements that are specifically tested for can also occur naturally in some plants and soil microorganisms and can generate false-positive results when using PCR. Several different methods described by Ahmed (2002) can be used to confirm PCR results: (i) restriction endonuclease digestion of the amplified product; (ii) hybridisation of the target sequence with specific DNA probes; (iii) sequencing of the PCR product; and (iv) nested PCR, where two sets of primer pairs specifically bind to amplified target sequences.

Due to the extreme sensitivity of qualitative detection methods, the aspect of thresholds might make the labelling of GMO products and its control more practical. Detection systems must therefore give quantitative answers (Schreiber, 1999). QC-PCR has been in practice for a few years now and Studer *et al.* (1998) have described the first application in the determination of GMOs in food. In response to EU regulations, the labelling of a GMO product could have an exhaustive approach. If validated qualitative methods do not detect GMOs in a product, the presence of protein would have to be tested for. The product is presumed to be not detectable when no protein is detected. A positive result through qualitative PCR qualifies the product as "non-approved GMO" and the level of GMO is then determined by validated qualitative PCR. Products are labelled as "non-approved GMO" if the level of GMO detected is more than an established threshold. A product needs to be labelled when the level of GMO detection is below the threshold (Yates, 1999).

Recently, the British government considered forcing the use of a "DNA bar coding" system on biotechnological companies. This patented technique would be able to identify GMOs. The principle behind this technique is that the same unique sequence must be added to all GMOs, regardless of how they were modified. This technique would not affect a plant's properties, for example, because the unique sequence would not encode for any protein. Furthermore, the addition of such a

sequence to a modified organism would most likely have no effect, since the genomes of most creatures are already filled with vast stretches of non-coding DNA. As a consequence of this technique, regulators would easily be able to detect GM food or crops that have been contaminated by GM strains.

As indicated previously, the detection of GM products is a difficult process because there is a need to have certain knowledge about the inserted DNA. It is necessary to either know the short sequences flanking any inserted piece of DNA, the sequence of the added DNA itself or the regions controlling the specific piece of added DNA. What complicates the detection of GM products even more is that biotechnological companies are often reluctant to cooperate in providing information about their technology for fear of other companies copying it. It could be that, in the future, companies would prefer a system such as DNA bar coding, because it would allow them to label their GM products without revealing any secrets. It would be possible for DNA bar codes to become lost or corrupted over many generations, but, in a field of plants, for example, it would not matter if a few plants lost their bar code. The Agricultural Biotechnology Council in Britain has already welcomed the idea cautiously, but only the future will tell if this concept will be implemented throughout the industry (Graham-Rowe, 2003).

Table 2.3 Summary of methods that specifically detect transgenic DNA or its products in foodstuff. Adapted from Ahmed (2002).

Parameter	**Protein-based**			**DNA-based**			
	Western blot	ELISA	Lateral flow strip	Southern blot	Qualitative PCR	QC-PCR and limiting dilution	Real-time PCR
Ease of use	Difficult	Moderate	Simple	Difficult	Difficult	Difficult	Difficult
Needs special equipment	Yes	Yes	No	Yes	Yes	Yes	Yes
Sensitivity	High	High	High	Moderate	Very high	High	High
Duration	2 days	30-90 min	10 min	6 h	1.5 days	2 days	1 days
Gives quantitative results	No	Yes	No	No	No	Yes	Yes
Suitable for field test	No	Yes	Yes	No	No	No	No
Employed mainly in	Academic labs	Test facility	Field testing	Academic labs	Test facility	Test facility	Test facility

There are no official standards for what constitutes a GM food or what makes a specific product GM free. Some detection tests are based on DNA analysis, while others focus on the gene product or protein. The different tests can vary from a three- to five-minute in-the-field test to more complicated laboratory investigations. A

so-called dipstick test is the fastest and easiest qualitative method. It is available in a kit and costs about $6 per test. Quantitative protein tests cost more, while DNA tests are very expensive, at about $250 per sample (Thomson, 2002).

Companies such as Genetic ID in the USA claim that they can detect DNA fragments as tiny as 80 to 120 nucleotides in almost any foods, including highly refined multi-ingredient products (Fagan, 1999). Thomson (2002) stated that the Council for Scientific and Industrial Research (CSIR) in South Africa can detect as little as 0.1% of transgenes in food products.

It will be impossible to show that food products are absolutely GM free, because it would then be necessary to test for every possible GM product. These food products will have to be continuously re-evaluated as assessment methods become more sensitive. In future, it will be important to establish a standard or definition that will determine when a food product is GM free (Thomson, 2002). Other key questions for the future are to determine how many tests will be necessary to identify GMOs in food and drink unequivocally and how to improve the efficiency of the available routine analysis (Meyer, 1999).

2.10 THE IMPORTANCE OF *S. CEREVISIAE* AND ITS ROLE AS A GMO

Yeast plays an important role as a model organism in the fields of biochemistry, genetics and molecular biology. Since yeast was first genetically transformed in 1978 (Hinnen *et al.*, 1978), it has been utilised as one of the most useful eukaryotic microorganisms for biological analyses and heterologous protein production (Russo *et al.,* 1995). Yeast strains belonging to *Saccharomyces* are the preferred organisms for the production of heterologous proteins, especially those meant for human therapeutic applications. The reason for this is that eukaryotic yeasts are more likely to produce proteins that are properly folded, because they are able to perform posttranslational modifications such as glycosylation (Gellissen *et al.*, 1992).

S. cerevisiae is undoubtedly the most important commercial microorganism with GRAS (Generally Regarded As Safe) status. It can boast a long history in the fermented food and beverage industry and today it is still brewing our beer, leavening our bread dough and sparkling our wine (Pretorius, 2000). Our knowledge of *S. cerevisiae* has improved even as far as the discovery of its complete genome sequence in 1996 (Goffeau *et al.*, 1996; Goffeau, 2000) and it serves as a model microorganism for studying other eukaryotic organisms. Its presence in clinical conditions is quite rare and it is generally considered to be relatively non-pathogenic (Chandra *et al.*, 2001). With the rapid development of molecular genetics and biology, *S. cerevisiae* has again become one of the pioneer microorganisms in the exploitation of one of mankind's most recent and profound revolutions, genetic engineering. *S. cerevisiae* was the first GMO cleared for use in food production as a baking and brewing yeast strain (Walker, 1998). Transgenic strains of *S. cerevisiae* were also used to produce the first approved human vaccine (against hepatitis B)

and food additive (calf chymosin in cheese production) through recombinant DNA technology (Barr *et al.*, 1989).

S. cerevisiae became known as the "wine yeast" due to the fact that it is universally preferred for initiating wine fermentations (Pretorius, 2000) and can be found almost exclusively in artificial, human-made environments (Vaughan-Martini and Martini, 1987). Impressive progress has been made in the development of molecular techniques for this yeast over the past 20 years (Dequin, 2001). During the 1990s, substantial effort, entailing mainly recombinant DNA approaches, went into the development of new wine yeast strains (Pretorius and Van der Westhuizen, 1991; Barre *et al.,* 1993; Butzke and Bisson, 1996; Querol and Ramon, 1996; Henschke, 1997; Pretorius, 2000). Some of the most remarkable advances were improved process performance, off-flavour elimination, increased by-product formation, improved hygienic properties and increased substrate utilisation (Dequin, 2001).

The origin of *S. cerevisiae* is controversial. Surprisingly, and in contrast to what many people believe, ecological evidence based on direct isolation procedures without any enrichment effect clearly indicate that fermentative species of *Saccharomyces*, e.g. *S. cerevisiae*, can hardly be found on healthy, undamaged grapes. They are also rarely isolated from vineyard soils (Martini, 1993). In general, *S. cerevisiae* occurs at concentrations of less than 10-100 CFU/g of grapes (Fleet *et al.*, 2002). Nevertheless, there is a belief that the primary source of *S. cerevisiae* is the vineyard and that its occurrence differs entirely with each plant and grape cluster (Török *et al.*, 1996). *S. cerevisiae* is not seen as an airborne contaminant, because plates never become contaminated with strains of *S. cerevisiae*. Therefore, this yeast needs a vector to reach the grapes. Mortimer and Polsinelli (1999), for instance, proposed that damaged grapes berries are rich depositories of microorganisms, including *S. cerevisiae*, and that these microorganisms are inoculated into the interior of damaged berries through insects. It is unknown whether one or more species of insects are the principal vectors of microorganisms to damaged berries. It was found that, even during the winter months, honeybees had a high percentage of *S. cerevisiae* on their bodies, while wasps carried the yeast at a much lower frequency (Stevic, 1962). Several studies have also shown that *Drosophila* carry an array of yeast species, including *S. cerevisiae* (Phaff and Knapp, 1956; Phaff *et al.*, 1956a; 1956b; Lachance *et al.*, 1994).

Another school of belief is that a natural origin for *S. cerevisiae* should be excluded and that this species originated from the hybridisation of other *Saccharomyces* species that were then selected in manmade environments. Evidence indicated a direct association of *S. cerevisiae* with manmade and artificial environments such as wineries (Martini, 1993). Typically, an abundance of *S. cerevisiae* can be found on the surfaces of winery equipment, forming an important part of what is called the "residential" or "winery" yeast flora (Fleet and Heard, 1993). The foam formed at the top of some open fermentations is also rich in

S. cerevisiae, which may be transported by air currents in the cellar (Mortimer and Polsinelli, 1999). Any occurrence of *S. cerevisiae* in vineyards would then be the result of back transportation by insects from the cellars to the vineyards (Naumov, 1996).

S. cerevisiae has seldom been isolated from areas less closely associated with humans (Naumov and Naumova, 1991; Naumov, 1996; Naumov *et al.,* 1998). Sniegowski *et al.* (2002) provided three explanations for the presence of *S. cerevisiae* in natural habitats that presently cannot be ruled out: (i) *S. cerevisiae* was domesticated and occasionally established synanthropic natural populations; (ii) a wild population of *S. cerevisiae* has recently expanded its range from a single region of origin (Naumov and Nikonenko, 1988), possibly in association with humans; and (iii) there are diverse, wild populations of *S. cerevisiae* that are globally distributed and have existed independently all along, predating domestication. Naturally, combinations of these scenarios are also possible, e.g. ongoing gene flow between domesticated and wild populations of *S. cerevisiae* cannot be excluded.

Although laboratory yeasts originated from industrial yeast (Mortimer and Johnston, 1986), they have special features, e.g. they are usually isogenic, they are haploid of either the a or α mating type, they can sporulate in the diploid form and they have multiple auxotrophic mutations. Industrial yeasts, on the other hand, do not have any of these features that are necessary for molecular analyses and gene cloning. The specific genetic manipulation systems for laboratory yeast are not always applicable to industrial yeast, because they are genetically diverse, usually diploid or polyploidy, with very weak or no sporulation competence, and prototrophic (Randez-Gil *et al.*, 1999; Pretorius, 2000; Dequin, 2001).

Traditionally, the genetic improvement of industrial yeast strains relied on classical genetic techniques, such as mutagenesis, hybridisation, protoplast fusion and cytoduction. For wine strains, techniques like these were usually followed by the selection of broad traits, such as fermentation capacity, absence of off-flavours and ethanol tolerance. Other traits that are selected for are fast dough fermentation, osmotolerance, rehydration tolerance and organic acid resistance for baker's strains, and flocculation and carbohydrate utilisation in the case of brewer's yeast (Dequin, 2001).

The demand for more specialised wine yeast strains has been increasing in the past decade and, as a result, the number of commercialised, selected strains has increased from about 20 to more than 100 (Dequin, 2001). Although many wine yeast strains with new properties have been generated through classical genetic techniques (Barre *et al.*, 1993), only a few of these strains have been commercialised. Today, the majority of commercial wine yeasts are strains of *S. cerevisiae,* which are characteristically predominantly homothallic, and diploid/aneuploid with low sporulation ability (Bidenne *et al.*, 1992; Rachidi *et al.*, 2000). One of the major shortfalls of the classical genetic techniques has always been the difficulty of adding or removing features from a strain without changing its

performance. Therefore, one of the major advantages of genetic engineering over classical genetic techniques is that only one characteristic can be precisely altered without other desirable characteristics being lost in the process (Dequin, 2001). The combination of techniques such as tetrad analysis, replica plating, mutagenesis, hybridisation and recombinant DNA methods has already increased the diversity of yeast immensely. By using procedures such as gene cloning and transformation, the possibility exists to alter wine yeast characteristics with extreme precision. Through these existing properties, strains can be modified, new characteristics can be introduced without negatively affecting other desirable properties and unwanted traits can be totally eliminated (Pretorius, 2000).

One of the most important biotechnological challenges in wine yeast is the improvement of the traits that play a role in must fermentation. The application of molecular techniques to the study of wine fermentation provides a useful tool for the genetic improvement of wine yeast strains. These yeasts provide more of a challenge, since their prototrophic and homothallic nature and higher degree of ploidy necessitate dominant selectable markers for the successful transformation of such strains (Kozovska *et al.*, 2001). There are several dominant drug-resistance markers for use in *S. cerevisiae* (Van den Berg and Steensma, 1997). The G418 resistance marker (Wach *et al.*, 1994) and another dominant selection marker based on resistance to the sulfonylurea-herbicide, sulfometuron-methyl (SM), are especially widely used (Xiao and Rank, 1989). The latter method of dominant selection provides an integration site close to the *ILV2* locus in the genome of *S. cerevisiae*, which encodes for the enzyme, acetolactate synthase. Several drug-resistance markers that were tested with industrial yeast strains are listed by Akada (2002).

As early as the beginning of the 1980s, brewers took an active interest in yeast genetics, which resulted in the production of hybridised and genetically modified yeasts with improved characteristics (Hinchliffe, 1992; Hammond, 1995; Benitez *et al.*, 1996). Yeast biotechnology especially offers the profitable use of different resources. In future, baker's yeast could be propagated on new raw materials, such as starch, cellulose wastes or cheese whey, which will result in cheaper, more reliable procedures than the traditionally used methods. The need for baking additives can also be eliminated by baker's yeast expressing heterologous enzymes, creating a better quality end product and increasing profit. Tailor-made enzyme cocktails could be expressed specifically for any baking requirement. In addition, hypersensitivity to allergens associated with baking might also be reduced through yeast biotechnology (Randez-Gil *et al.*, 1999).

For the commercial application of an industrial yeast strain it is important that all unwanted DNA (antibiotic-resistance markers, yeast drug-resistance markers) introduced into the yeast are eliminated. Several counter-selection systems developed for this purpose are listed by Akada (2002). In those cases where recombinant yeast contained no *E. coli* plasmid sequences (Fujii *et al.*, 1990; Yamano *et al.*, 1994; Marín *et al.*, 2001), no problem existed for the transfer of

drug-resistance genes to other organisms (Akada, 2002). However, even if the heterologous genes originated from food microorganisms, the gene products must be checked with respect to their toxicity and allergic effects (Yamano *et al.*, 1994; Saito *et al.*, 1996). An important aspect of heterologous gene expression in *S. cerevisiae* is that the gene products may not be identical to those from the original microorganisms due to protein modifications (e.g. glycosylation), which are known to be diverse (Gemmill and Trimble, 1999).

In their guidelines for GM food, the Japanese Government exempted "self-cloning" from the safety assessment of food microorganisms (Nakamura, 2001; Hino, 2002). With self-cloning, genes from microorganisms are cloned within the microorganisms and therefore self-cloning yeasts do not need to be treated as recombinant yeasts. Akada (2002) stated that the self/non-self cloning criterion will become very important for the commercial application of GM microorganisms in the future.

Despite remarkable progress over the last two decades, only two recombinant yeast strains have so far received official approval. In the early 1990s, the British Government gave official approval for a recombinant baker's and a brewer's yeast. The baker's yeast was derepressed for maltase and maltose permease (Aldhous, 1990) and the brewer's yeast expressed the *STA2* gene, which enabled it to produce exocellular glucoamylase (Hammond, 1995). In the case of the baker's yeast, a gene and promoter from a closely related yeast isolate were transferred to another strain that was well adapted to industrial culture and production. This yeast was thus an example of a self-cloned organism and immediately prompted discussion on the safety, regulatory and labelling issues regarding GMOs (Teuber, 1993; Lloyd-Evans, 1994). The safety precautions taken for the baker's yeast included the complete removal of foreign DNA, keeping synthetic DNA linkers to an absolute minimum, preventing any possibility of fusion protein expression and incorporating the construct into the yeast genome to obtain genetic stability. The yeast was also shown to behave similarly to the parent strain in the environment (Teuber, 1993).

Nevertheless, neither of these two yeasts is currently used commercially. The major obstacle to the commercialisation of GM industrial yeast strains is the public's acceptance. Except for some nutritional or hygienic advances, the benefits of GM yeast strains are mostly not perceptible to consumers (Dequin, 2001). Efforts will have to be made to increase the public's awareness of the potential benefits (e.g. safe production, high quality and low cost) of recombinant DNA technology. The presence (e.g. bread, wine) or absence (e.g. beer, filtered wines) of the GMO in the product will also be very important for the acceptability of GMOs in food and beverages. Detection methods that can differentiate between these two types of products will have to be developed. Aspects concerning the practical consequences of the introduction of this technology for the industry, e.g. the risks associated with the release of GMOs, should also be debated. In future, it might also be necessary

to separately consider each different industry, with specific approaches being defined and implemented for each (Dequin, 2001).

It is clear that the successful application of transgenic yeast, food and wine will eventually depend on several scientific, technical, economic, marketing, safety, regulatory, legal and ethical issues. Therefore, no unrealistic expectations can be harboured that GMOs will soon be commercialised. Dramatic breakthroughs can only be expected after the available technology, currently being applied to laboratory strains of *S. cerevisiae,* can be exploited on the much more complicated genomes of the industrial wines yeast strains (Pretorius, 2000). In the future, the implementation of functional genomics programmes will enable the achievement of various industrial objectives, e.g. the determination of the side effects of genetic alterations on functionality in the final products, the creation of pleiotropic effects by specific regulatory mutations, the prediction and improvement of stress responses and the directing of metabolic engineering efforts (Pérez-Ortín *et al.,* 2002).

2.11 CONCLUSION

The development of GMOs has opened up endless possibilities for basic research in genetics, but also, importantly, in the application of modern achievements in molecular biology to the consumer's benefit, subject to consumer acceptance and economic feasibility (Linko *et al.*, 1997). Although there is much talk about the costs and benefits of GMOs, it is important to keep the debate balanced.

The most prevalent issue raised by the opponents of GMOs is our poor knowledge about the effect of gene transfer on the environment. They are of the opinion that GMOs are an industrial advance profiting only a small part of the population and feel that the planet's resources should be better distributed. A climate of mistrust covers all GMOs, except those engineered for medical purposes. However, not all the issues associated with plants and around GMOs are relevant to microbial GMOs. The use of GMOs in the open market is still a big uncertainty because of consumer mistrust and tight labelling legislation that allows consumers to choose products on the basis of their content of GMOs or derived products. In general, regulation might not be the real problem regarding the acceptance of GMOs (Renault, 2002).

In light of the complexity of environmental risk assessment, a case-by-case approach supported by many regulatory systems is probably still the best way to proceed in the future (Hails and Kinderlerer, 2003). Scientific bodies should perform risk assessment and authorities should perform risk management. Wherever science does not provide convincing answers, the precautionary principle must be applied. In this way, the public's concerns will be addressed (Mahler, 1999).

Much of the research into risk assessment is either being performed inside the laboratory or in small-scale field experiments. A challenging aspect for the future will

be how to fit these research results to scales that will be relevant to the possible commercial release of GMOs (Hails, 2000).

Another important aspect for the future is that Europe could be in line to lose its science base if the current GMO opposition continues. With globalisation of the biotechnological industry, large corporations are tempted to move their interest and collaboration with researchers to countries where conditions are more favourable. European companies such as the Swiss agro-biotechnological giant, Novartis, have already invested significant amounts of money in the USA (Frank, 2000).

It is mostly the seed companies and producers that benefit from first-generation GM crops, but consumers will benefit when second-generation GM foods become available in supermarkets. Until that happens, Europe is unlikely to soften its position. European farmers are largely subsidised and it is not in their interests to import food from the USA (Thomson, 2002). In future, each country should seriously decide whether it wants to be isolated from the rest of the world by not exploring the new technology. Moratoriums on the commercial applications of GMOs might not be the answer, but stringent control and regulation by government would be a better option.

The road ahead to the full acceptance of genetically engineered food may still be difficult. It is evident that a rosy future for GM producers only lies in the willingness of consumers to accept and buy new GM varieties. The global benefits of GM products are reduced if consumers in some countries reject them. In rich countries, where consumers can indeed afford to be more critical of these new food varieties, consumers might simply not want to purchase them, regardless of how cheap these new products may be. In fact, there will always be a part of the population that will refuse to make use of new technologies. Despite the productivity gains in GM products, the market for non-GM foods may still increase (Nielsen *et al.*, 2003). Niche markets for non-GMO products could therefore grow in the future.

Niche markets for certain GMO products could also arise. One possible example could be the wine industry. If traditional grape growers and winemakers could be convinced of the organoleptic, hygienic or economic benefits of utilising transgenic grapevines and yeast, they would be in a strong position to implement the use of such GM commodities. Most of the wine enterprises form part of fully integrated agro-industries that could have direct control over the emergence of new, specialised niche markets for GM wine products. It is possible that GM wines produced by a limited number of interested producers could attract enough attention for the establishment of a successful niche market, since wine consumers in such niche markets are often passionate, well educated, informed and curious. Once the broader benefits of GM technology become more apparent, small niche markets could possibly evolve into a more general acceptance (Viviers and Pretorius, 2002). The emergence of "functional foods" or "nutraceuticals" may alter the terms of the GM debate, as the improvement of human health through the modification of foods (including wine) may become more acceptable to consumers (Gibbs, 2000).

For biotechnology to be a success factor in economic and social development of the future, it is vital that scientific awareness is promoted to the public. The rapid evolution of scientific knowledge has created so much innovation in the past few years that public opinion is filled with a sense of discomfort and anxiety regarding the future (Corda Mannino *et al.*, 1998). Most scientists believe that risks to the environment and to human and animal health are mainly hypothetical and that all the proper safeguards are in place. However, it is imperative that research continues, especially on the long-term effects of this technology (Thomson, 2002).

The variability that maintains evolution in organisms is created by the inherent capacity of the genome to reorganise itself in response to external and internal signals. By implication, genetic engineering cannot be made safer than biology itself. A defined biosafety baseline can be set as the limit for levels of tolerable damage by, and acceptable risk with, transgenic organisms. It may be possible to manage potential negative effects with good experimental and scientific practice. Our abundance of scientific knowledge about the evolutionary significance of genetic variation, together with our experience in technology, should confirm this (Käppeli and Auberson, 1998). In the near future, the statement that GMOs are organisms in which the genetic material has been changed in ways that do not occur naturally through natural recombination or mating, might become archaic. We will probably be surprised by genome evolution studies that will show us numerous examples of organisms in which the genetic material has been changed in ways that will be far too complex for humans to mimic (Renault, 2002).

As for the future of gene technology, it is an inevitable development. Many more countries will certainly continue to adopt gene technology legislation prescribing the evaluation of benefits to the community and the contribution to sustainable development. This new technology will find its natural place among all other tools that humans have mastered in the past as more useful applications for humanity come to the marketplace (Mahler, 1999). Hopefully future debates will be scientifically better informed, as well as ethically subtler, than has often been the case in the past (Polkinghorne, 2002).

Despite all the chemical scares, it is important to remember that life expectancy of the human race has been steadily increasing since the beginning of the previous century and that our food has never been as safe as it is today. The real food safety issues are those incidents of hygiene hazards, such as microbial contamination (Anklam and Battaglia, 2001), and not necessarily the specific GMO or the process by which the product has been made. Biotechnology has already changed the lives of more than 250 million people through innovative drugs and vaccines. In the past, when the future of recombinant DNA technology was in question, hope prevailed over fear and reasoning over sensationalism (Feldbaum, 2002). We must do the best we can to insure that history repeats itself in other important fields of biotechnology.

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CHAPTER 3

RESEARCH RESULTS

The detection and monitoring of genetically modified yeasts within a vineyard microbial population

3. RESEARCH RESULTS

3.1 INTRODUCTION

Since the beginning of recorded civilisation, the yeast *Saccharomyces cerevisiae* has been instrumental in planting many of the most important milestones on the road of the development of civilisation. Of all the microorganisms, it was the first to be (i) domesticated by people for the production of food (e.g. bread in ancient Rome in 100 BC) and beverages (e.g. beer and wine in Assyria, Caucasia, Mesopotamia and Sumer in 7000 BC), (ii) observed microscopically (by Antonie van Leeuwenhoek), (iii) described as a living biochemical agent of transformation (by Louis Pasteur), (iv) used as a host for the production of the first recombinant vaccine (against hepatitis B) and the first recombinant food enzyme (the milk coagulation enzyme, chymosin, for cheese making), and (v) used to reveal the entire nucleotide sequence of a eukaryotic genome (Pretorius *et al.*, 2004). It is therefore not surprising that today, *S. cerevisiae* is not only regarded as one of the most useful scientific model organisms for fundamental research, but also as the most important industrial organism with GRAS (Generally Regarded As Safe) status. *S. cerevisiae* is used in the annual production of 60 million tons of beer, 30 million tons of wine, 800000 tons of single cell protein and 600000 tons of baker's yeast, while its importance as a host organism for the production of commercially important enzymes, chemicals, therapeutic proteins and other pharmaceutical products is growing (Pretorius *et al.*, 2004). The demand for improved *S. cerevisiae* strains for a wide variety of existing and new products becomes greater and more urgent.

In recent years, considerable efforts have been made to improve baking, brewing and wine yeast strains through genetic engineering. The main targets for these strain development programs have been, and still are, the improvement of fermentation performance, processing efficiency, and sensory quality, as well as the development of new strains with reduced risks and enhanced benefits for health. Numerous stable genetically modified (GM) yeast strains already exist in laboratories, while many others are being constructed. Some of these GM yeasts that fully comply with the strict GM-related legislation of most countries have even been cleared by regulatory authorities for commercial use. For example, as early as the 1990s, GM baking and brewing strains received approval for commercial use. The GM baking strain contains constitutively expressed maltose permease and maltase genes that enable *S. cerevisiae* to produce CO_2 faster than conventional baker's yeasts, thereby ensuring that dough rises more rapidly (Smith, 1998). The GM brewer's yeast expresses a glucoamylase-encoding gene that allows partial hydrolysis of wort maltodextrins, thereby yielding a lower-carbohydrate beer (Smith, 1998). More recently, a wine yeast has been developed that is capable of converting malic acid in grape must either to ethanol (malo-ethanolic fermentation) or to lactic acid (malolactic fermentation) during alcoholic fermentation of the winemaking process (Pretorius,

2003). These malo-ethanolic and malolactic wine yeasts negate the necessity of using lactic acid bacteria, which often lead to problems with sluggish or stuck malolactic fermentations and the production of biogenic amines in wine. The "malolactic yeast" was the first wine yeast to be commercialised by a yeast manufacturing company and it has been trialled in 2002/2003 in Moldavia. This represents the first large-scale (20000-litre) winemaking trial with a GM wine yeast (Pretorius and Høj, 2005).

With the benefit of hindsight, however, regulatory authorities appeared more willing to approve the use of the aforementioned GM baking, brewing and wine yeasts than the public is to use them (Pretorius, 2000). However, the application of genetic engineering, particularly in agriculture and food production, remains controversial. Countless reports on the topic have been published, and the arguments for and against the use of genetic modification, be they of an ethical, economic, environmental or otherwise scientific nature, have been made extensively in debates all over the world. Political bungling regarding some sensitive, public health-related issues and a rather silent scientific community, has created a negative public perception of GM-technology, particularly in European countries (Bauer *et al.*, 2004).

Given the current deeply rooted concerns of consumers and traditionalists, and a business environment in which product-liability litigation is a grim reality in the market place, the general view in the wine sector is that it would be commercial suicide to market the first GM wine (Pretorius and Høj, 2005). Therefore, the future use of GM wine yeasts hinges on a scientifically sound evaluation of the safety and of the potential environmental and economic impact of genetically manipulated organisms (GMOs) (Bauer *et al.*, 2004). The latter evaluation requires the study of complex interactions and ecosystems, and needs to assess a large number of interrelated parameters.

Such risk assessments will typically integrate several multidisciplinary approaches, and should include, among others, ecologists, microbiologists, geneticists and biochemists. It is probably due to this intrinsic complexity of the topic that few studies have been conducted previously to holistically assess the environmental impacts of GMOs (Bauer *et al.*, 2004). Such a holistic assessment would need to include investigations that address several unresolved issues. These issues include the following: (i) the evaluation of available detection methods for GM yeast; (ii) the assessment of the spreading of existing commercial wine yeast strains in the environment; (iii) the comparison of the behaviour of parental yeasts and their GM derivatives in model systems to assess whether GM strains may possess a selective advantage which could lead to their spreading; (iv) the evaluation of the probability of transgenes spreading vertically to other yeast strains or horizontally to other species; and (v) assessment of the consequences of genetic modification on the modified yeast itself (Bauer *et al.*, 2004).

To this end, a number of laboratories developing wine yeasts have decided to

work together to address these issues. In evaluating all available methods for the detection of GM yeast, the most common techniques used to detect GMOs in food can find application to trace GM yeast in a vineyard or wine environment. These methods include Southern blotting and PCR analysis for the detection of transgenic DNA and immunological tests for the detection of GM proteins (Ahmed, 2002). However, in the assessment of complex communities, none of these techniques offers easily reproducible results, suitable for standard analysis in a wine cellar environment (Bauer *et al.*, 2004).

The assessment of the survival and spread in nature of existing commercial wine yeast strains is imperative, since these yeasts will be targeted for genetic manipulations. Data from groups in South Africa, France and Portugal suggested that commercial wine yeast strains are not easily spread from the cellar to the vineyard (Bauer *et al.*, 2004).

An objective of a recently-launched study is to investigate the probability of transgenes spreading vertically by monitoring sporulating cells in wine fermentations by means of fusing a Green Fluorescent Protein (GFP) to a strong sporulation-specific gene. In that study, the occurrence of horizontal transfer will be assessed by monitoring industrial wine yeast strains transformed with dominant selection markers under "worst case scenario" conditions, which might be optimal for horizontal DNA transfer to occur (Bauer *et al.*, 2004).

Regarding the issue of assessing the consequences of genetic modification on the modified yeast itself, several studies are underway to analyse the transcriptomes in parental and GM yeast strains through microarrays. This will lead to the establishment of databases where various metabolic consequences of genetic modifications can be analysed (Bauer *et al.*, 2004).

The research described in this chapter is focused on the issue of comparing the behaviour of specific parental and GM yeast strains in model systems in order to determine whether the GM strains have any selective advantage, which could lead to their disseminating and becoming ecologically established. In view of the necessity to assess the fate of specific GM industrial yeast strains in natural ecosystems, a study was initiated to monitor their dissemination and survival on grapevines cultivated in a biologically contained glasshouse. Three strains of a well-known industrial wine yeast, *S. cerevisiae* VIN13, that have been genetically engineered to be amylolytic, glucanolytic and pectolytic were the GM yeasts of choice in this study. This is the first report of the assessment of the fate of GM strains of VIN13 that are suitable for the wine and baking industry. The occurrence of native yeast populations on the grapevines was also assessed. This research was conducted at the Forschungsanstalt Geisenheim in Germany and the Department of Microbiology and the Institute for Wine Biotechnology at the University of Stellenbosch, South Africa.

3.2 MATERIALS AND METHODS

3.2.1 GLASSHOUSE SET-UP

The glasshouse was situated at the Forschungsanstalt Geisenheim, Geisenheim, Germany and contained 80 one-year-old *Vitis vinifera* Riesling KI 110 Gm/SC grapevines, which were subjected to normal viticultural practices. In 1999, the first year of monitoring, the grapevines were not divided into blocks since no GM yeast was inoculated. In 2000 and 2001, the grapevines were divided into four blocks consisting of 20 grapevines each and each block was treated differently.

3.2.2 MIST INOCULATION OF GM YEASTS ONTO GRAPEVINES

The permit for the inoculation of the GM yeasts in the secluded glasshouse environment was granted in June 2000. Inoculation was performed once-off during August of both 2000 and 2001. See Fig. 3.1 for the glasshouse set-up in 2000 and 2001 and the inoculation of the different GM yeasts. All the yeasts were cultured in 2500 ml YPD broth (containing 1% yeast extract, 2% glucose, 2% peptone from casein) at room temperature (20°C) to an optical density (OD) of 6 to 7 at 600 nm, after which they were centrifuged for 5 min at 3000 rpm. The pellets were washed once with sterile water and again resuspended in water. Dilutions were made in such a way that 1.5 litre of washed GM yeast culture was inoculated at a concentration of 2.0E+06 colony forming units (CFU)/ml into each block consisting of 20 grapevines. The yeasts were mist-inoculated onto the selected grapevines with plastic spray bottles, while the other treated blocks of grapevines were covered with plastic sheets to prevent cross contamination. The plastic applicator was moved slowly over the grapevines until they were wet to runoff. About 75 ml of yeast culture was inoculated onto each plant. The precise concentration and ratio at which each yeast was inoculated are indicated in Table 3.1.

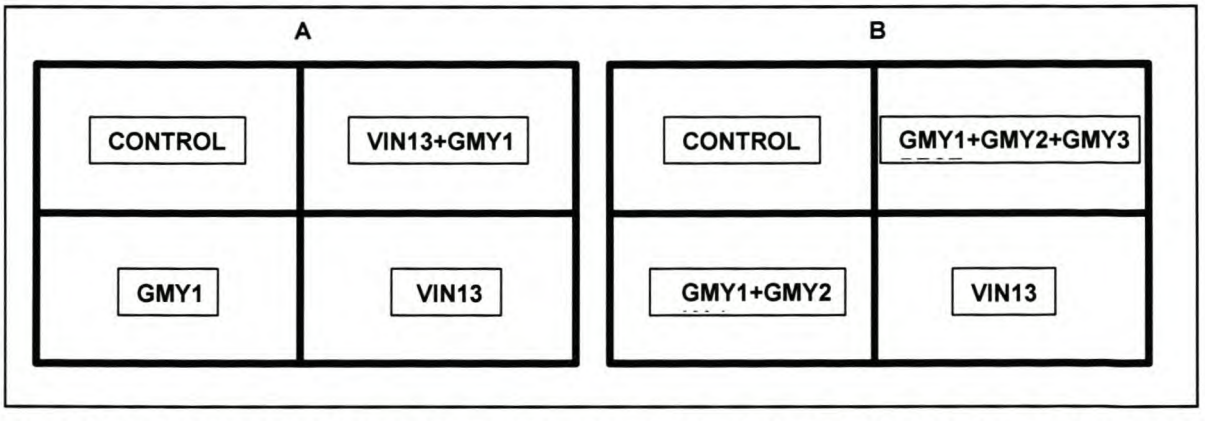

Fig. 3.1 The arrangement of the 80 grapevines in the secluded glasshouse into four blocks consisting of 20 grapevines each. In 2000 (A), when only one GM yeast strain, GMY1, was inoculated, the organisation of the glasshouse differed from the organisation of the glasshouse in 2001 (B), when three different GM yeasts, GMY1, GMY2 and GMY3, were inoculated.

3.2.3 GM YEASTS INOCULATED

3.2.3.1 GMY1

In 2000 and 2001, a well-known industrial wine yeast strain of *S. cerevisiae,* VIN13, expressing an extra gene, *LKA1*, was inoculated onto the grapevines as shown in Fig. 3.1. This GM yeast was designated as GMY1. *LKA1* was originally cloned from *Lipomyces kononenkoae* and encodes a raw starch-degrading α-amylase that liberates reducing sugars from glucose polymers containing both α-1,4 and α-1,6 bonds (Steyn *et al.,* 1995). It was introduced into VIN13 on an integrating plasmid via the sulfometuron methyl resistance marker *(SMR1)* under the control of the phosphoglycerate kinase (*PGK1)* promoter and terminator (Gundllapalli Moses *et al.*, 2002).

Table 3.1 The concentrations and ratios at which the different yeasts (VIN13 and GM yeasts) were mist-inoculated onto the grapevines in 2000 and 2001.

2000	CFU/ml	2001	CFU/ml
VIN13	2.4E+06	VIN13	2.0E+06
GMY1	2.9E+06	GMY1:GMY2 1:1	2.0E+06:2.0E+06
VIN13:GMY1 1:1	2.2E+06:2.2E+06	GMY1:GMY2:GMY3 1:1:1	2.0E+06:2.0E+06: 2.0E+06

3.2.3.2 GMY2

In 2001, VIN13 expressing two extra genes, *end1* (encoding endo-β-1,4-glucanase) and *XYNC* (encoding endo-β-xylanase), was inoculated onto grapevines according to the plan shown in Fig. 3.1. The endo-β-1,4-glucanase gene (*end1*) originates from *Butyrivibrio fibrisolvens* H17c and the endo-β-xylanase from *Aspergillus niger*. They were introduced into VIN13 on an integrating plasmid via the *SMR1* gene under the control of the alcohol dehydrogenase (*ADH1)* promoter and terminator. This strain was designated GMY2 (Strauss, 2003).

3.2.3.3 GMY3

In 2001, VIN13 expressing two genes, *pelE* (encoding pectate lyase) and *peh1* (encoding polygalacturonase), was inoculated onto grapevines as shown in Fig. 3.1. The pectate lyase-encoding gene (*pelE*) originates from *Erwinia chrysanthenii* EC16 and the polygalacturonase-encoding gene (*peh1*) from *Erwinia carotovora* subsp. *carotovora*. Both of these genes were introduced into VIN13 on an integrating plasmid via the *SMR1* gene under the control of the alcohol dehydrogenase (*ADH1)* promoter and terminator. This strain was designated GMY3 (Strauss, 2003).

3.2.4 ISOLATION OF YEASTS FROM LEAVES AND GRAPES

Yeast populations were monitored seasonally from 1999 to 2001 as soon as the leaves began sprouting. Weekly sampling commenced in May/June and continued until the end of October/November of each year. At first, only leaves were sampled, until the berries ripened in June/July. Towards the end of the season, only berries were sampled when the leaves started to dry out. In 1999, ten grapevines were randomly picked and sampled each week to determine a baseline of native yeasts present on the grapevines. In 2000 and 2001, three different grapevines were randomly chosen from each of the four blocks each week. Four leaves were sampled from each vine: one each from the bottom and top and the sunny and shady sides of the vine. The leaves from the bottom and top were shredded by hand and washed together for 60 min at 120 rpm in 50 ml of 0.9% NaCl containing a drop of Tween 80, after which the washing buffer was centrifuged at 3000 rpm for 15 min. The washing step was then repeated once with 40 ml of 0.9% NaCl. The leaves from the sunny and shady sides of the vine were treated in the same way. Pellets were suspended in a chosen volume of sterile water or 0.9% NaCl and spread plated onto YPD, YM (0.3% yeast extract, 1% glucose, 0.3% malt extract, 0.5% peptone, 2% agar), and YM agar plates supplemented with 12% ethanol. Plates were incubated for two to three days at room temperature (22°C). Biphenyl (0.04%) or sodium propionate (0.2%) was incorporated into the plates for the inhibition of fungal growth. Each different type of yeast-like colony was enumerated and streaked onto YPD plates for further identification.

As soon as the berries ripened, they were also sampled on a weekly basis, washed in 30 ml of 0.9% NaCl and treated in the same manner as the leaves. From each randomly sampled grapevine, 12 berries were picked: three each from the bottom and top and three each from the sunny and shady side of each vine. Later in the season, when berries became depleted, eight berries were sampled from each selected grapevine. In the sampling season of 2001, only eight berries were constantly sampled, since the grapevines were still growing in pots and produced fewer grapes each year.

3.2.5 ISOLATION OF YEASTS FROM THE SOIL AND BARK

Soil samples and pieces of bark were monitored at intervals during autumn and winter for the presence of the GM yeasts. Soil sampling commenced in September 2000 and was repeated three times in October 2000, once in each of November 2000, January 2001, February 2001 and April 2001, twice in May 2001 and September 2001, three times in October 2001, twice in November 2001, once in December 2001, three times in January 2002 and once in February 2002. With each sampling of the soil and bark, three plants were randomly chosen from each block. From each pot, 10 g of soil was aseptically collected with a spatula and added to 90 ml of Ringers solution (Merck) in an Erlenmeyer flask. The flasks were vigorously shaken by hand for 2 min and by vortex (Vortex Genie 2, Scientific Industries) for

1 min at top speed. Serial dilutions were made and spread plated onto YM plates containing 12% ethanol. The plates were incubated for two to three days at 30°C and replica plated onto Lysine medium (0.67% YNB without amino acids, 0.08% lysine monohydrate, 5% glucose, 2% agar, pH 6.5) and the different assay media pertaining to each GM yeast. See section 3.2.6 for a description of the assay media.

Sampling of the bark was done twice in November 2001, once in December 2001 and twice in January 2002. From each selected plant, 0.3 g of bark was collected and vortexed for 1 min in 9 ml of Ringers solution. It was not possible to collect more bark from these young grapevines. Once in January 2001, before the grapevines were pruned, small (2 cm) pieces of vine branches were sampled from the inoculated blocks and tested for the presence of GMY1. A few branches (8-10) were added to 9 ml of Ringers solution and vortexed for 1 min. The bark and branches were further treated in the same manner as above.

3.2.6 YEAST ASSAYS

All isolated yeast-like colonies were first replica plated on Lysine medium. Yeasts that did not grow on Lysine media were considered to be *S. cerevisiae* and further tested on the different differential indicator media.

3.2.6.1 Assay for α-amylase production by GMY1

The production of α-amylase was assessed by replica plating colonies onto Phadabas starch plates containing 0.67% YNB with amino acids, 0.1% glucose, 20 Phadabas tablets/litre (Pharmacia and Upjohn) and 2% agar. The plates were incubated at 30°C for three to four days. Colonies showing α-amylase activity were identified by clear zones of starch degradation (Fig. 3.2).

Fig. 3.2 A Phadabas plate showing α-amylase activity by GMY1 (indicated by LKA1) through a clear zone of starch degradation. VIN13 expresses no α-amylase.

3.2.6.2 Assay for glucanase activity by GMY2

Colonies were grown on normal YPD screening plates containing either 0.3% viscosity carboxymethylcellulose (Sigma), 0.1% barley β-glucan (Sigma), or 0.4% lichenan (Sigma). After two days of incubation at 30°C, colonies were rinsed off with distilled water before the plates were stained with 0.1% Congo Red. If viscosity carboxymethylcellulose was used, the plates were destained with 1M NaCl. Colonies showing glucanase activity were identified by clear zones around the colonies (Van Rensburg *et al.*, 1997).

3.2.6.3 Assay for polygalacturonase activity by GMY3

Cultures grown overnight in YM medium were spotted onto screening plates containing 1.25% polygalacturonic acid (Orange, Sigma), 0.68% potassium phosphate pH 3.5, 0.67% YNB without amino acids, 1% glucose, 0.13% amino acids and 2% Bitek agar. After five days of incubation at 30°C, colonies were rinsed off with distilled water and the plates were infused with 6M HCl to visualise the zones. HCl precipitates polygalacturonic acid to form a white background (McKay, 1988).

3.2.7 IDENTIFICATION OF YEASTS

Representative yeast colonies from the plates were streaked onto YM agar for further isolation and identification. Each non-*Saccharomyces* isolate was re-streaked onto Wallerstein Laboratory Nutrient medium (WL) (Difco) for the observation of colony morphology and incubated at 30°C for four to five days. Yeast strains display different colour and colony morphology on WL agar, a medium that was designed for use in the brewing and industrial fermentation industries to observe microbial populations (Green and Gray, 1950). Isolates displaying the same colony morphology on WL medium were considered to be identical and a few representatives from each colony type were further analysed. Isolates were identified according to the principal method of Kurtzman and Robnett (1997; 1998). The principle of this method is that most yeast species can be identified from sequence divergence in the D1/D2 domain of the 26S rRNA gene, which is located in the first 600 bases region of the 26S rRNA gene. Isolates were maintained on YM slants and stored at 4°C.

3.2.7.1 Genomic DNA isolation

About 1.5 ml of overnight cultures in YM medium was centrifuged at 3000 rpm for 2 min and the cells were resuspended in 200 µl of breaking buffer (containing 2% Triton-X 100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8). After the addition of 0.3 g glass beads (0.5 mm diameter) and 200 µl of PCI (phenol:chloroform:isoamylalcohol = 25:24:1), the mixture was vortexed for 4 min. Then, 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) was added, gently

mixed and centrifuged for 5 min at 13 000 rpm. DNA was precipitated with ethanol and treated with RNase (10 μg/ml) in 50 μl of TE buffer.

3.2.7.2 PCR and sequencing

Genomic DNA was amplified by polymerase chain reaction (PCR) using a Perkin Elmer GeneAmp PCR system 2400 with primers F63 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and LR3 (5'-GGTCCGTGTTTCAAGACG-3'), as indicated in Kurtzman and Robnett (1997; 1998). Each PCR reaction was performed in a total volume of 50 μl and contained 2 μl of genomic DNA, 2.5 μl of 10 μM of each primer, 25 μl of PCR Master Mix (containing 400 μM of each dNTP, 2 mM of $MgCl_2$ and 50 units/ml of *Taq* DNA Polymerase; Promega) topped up with water. Amplification was performed as follows: 2 min at 95°C; 30 cycles at 94°C for 1 min; at 57°C for 1 min and 72°C for 2 min. Final extension was at 72°C for 10 min. PCR products were purified using the Nucleospin® Extract Purification Kit (Macherey-Nagel) according to the manufacturer's instructions, and sequenced by an ABI Prism 3100 Genetic Analyser.

3.2.8 DISTINGUISHING BETWEEN WILD *S. CEREVISIAE* AND VIN13

In the blocks in which VIN13 had been inoculated, sequence polymorphisms of *S. cerevisiae* isolates were determined according to the method of Ness *et al.* (1993) to distinguish any wild *S. cerevisiae* isolates from VIN13. Many repeated sequences exist in *S. cerevisiae*, e.g. δ elements. These sequences are direct repeat elements of 0.3 kb flanking the TY1 retrotransposon and, statistically, one δ element can be found every 150 kb. The δ sequences are often concentrated in genomic regions adjacent to the tRNA genes and the δ elements, either directly or inversely repeated, are therefore separated by amplifiable genomic spacer regions. The position and/or the number of δ elements are different between strains. The amplification of yeast genomic DNA with primer δ1 (5'CAAAATTCACCTATA/TTCTCA3') and primer δ2 (5'GTGGATTTTTATTCCAACA3') yields amplified sequence polymorphisms, which are strain specific and stable under the classical conditions of cell multiplication. Even if the number of δ sequences is not variable between strains, a slight difference in the position of a band is sufficient to generate a distinguishing polymorphism.

PCR reactions were performed under the following conditions: 50 μl of reaction mixture was prepared with 5 μl of template, 1 μl of each primer (100 pmol/μl), 4 μl of dNTPs mix (10 mM of solution: 10 mM of dATP, 10 mM of dCTP, 10 mM of dGTP and 10 mM of TTP, Sigma), 5 μl of 10×NH_4 Bioline buffer without $MgCl_2$, 2 μl of $MgCl_2$ (50 mM Bioline solution), 0.5 μl of Biotaq polymerase (Bioline) and adjusted to 50 μl with water.

The programme used was that set out by Ness *et al.* (1993), with some modifications: an initial denaturing step at 95°C for 10 min; 95°C for 30 s, 45°C for 30 s and 72°C for 2 min (cycled 34 times); and a final extension at 72°C for 5 min before the samples were stored at 4°C.

3.2.9 TESTING OF FUNGICIDE

The following yeast strains were tested for their sensitivity to the fungicide, Ridomil (Syngenta), which has metalaxyl as the active ingredient: VIN13, GMY1, GMY2, GMY3 and a wild *S. cerevisiae* isolate. The yeasts were tested at an initial concentration of 1.36E+08, 9.9E+07, 1.01E+08, 1.5E+08 and 1.3E+08 CFU/ml, respectively. Every yeast culture was incubated at room temperature (20ºC) with a 0.15% and 0.3% Ridomil solution at a ratio of 20:1. Yeast viability counts were done after three, seven and 15 days of incubation. Ridomil was sprayed onto the grapevines at a concentration of 0.15% according to normal viticultural practices.

3.3 RESULTS AND DISCUSSION

3.3.1 YEAST DYNAMICS

3.3.1.1 Basal level of wild yeasts isolated in 1999

In 1999, the first year of sampling, yeasts were isolated in order to establish the basal level of yeasts present on the grapevines. No inoculation of GM yeast was performed in this year. Although ten plants were randomly sampled each week, yeasts were not always isolated from every plant. Most of the sampling was performed on leaves and berries, although only the leaves were sampled early in the season when the berries were not ripe enough (May to June). Late in the season, when the leaves were starting to dry out, mostly berries were sampled. Yeast numbers were determined as an average of yeast isolates from plants sampled per month and expressed as CFU/100 ml/leaf or berry. The colony forming units on the leaves and berries were specifically determined by washing two leaves and ten (or eight, depending on the seasonal stage) berries from each sampled grapevine in 50 and 40 ml of 0.9% NaCl, respectively, and converting it to 100 ml.

From May until October 1999, samples were taken for 19 weeks and 91 yeast isolates representing 10 genera were isolated. Mostly leaves were sampled, since the one-year old grapevines did not bear many berries. More yeasts were therefore isolated from the leaves. Only leaves were sampled during May, June and August 1999. There was only one week of sampling in May 1999 and consequently there were few yeasts isolated in that month.

Yeasts isolated from the leaves were identified as *Rhodotorula, Hanseniaspora uvarum, Yarrowia lipolytica, Saccharomycodes ludwigii, Kloeckera apiculata, Kluyveromyces lactis, Debaryomyces hansenii, Pichia* spp., *Candida* spp. and *S. cerevisiae* (Fig. 3.3A). All the *Candida* and *Pichia* spp. were indicated collectively, since some of these species were isolated in very low numbers. Several of the yeasts were frequently isolated from the leaves and berries at averages of more than 1.0E+03 CFU/100 ml/leaf or berry. *Rhodotorula, Y. lipolytica, Saccharomycodes ludwigii, Kluyveromyces lactis, Pichia* spp. and *Candida* spp. were isolated from the leaves most frequently (for three months or more) and at monthly averages of more

than 1.0E+04 CFU/100 ml/leaf. In August 1999, *Y. lipolytica* was isolated at the highest average of 2.0E+05 CFU/100 ml/leaf.

Except for *Rhodotorula, Kluyveromyces lactis* and *Kloeckera apiculata,* the same yeasts isolated from leaves were isolated from the berries (Fig. 3.3B). Yeasts from the berries were detected at a lower frequency and only *H. uvarum* was isolated in more than one month. The fact that it was the first year of production for the grapevines, and fewer berries could be sampled, may explain the lower number of isolates from the berries. *Y. lipolytica, Saccharomycodes ludwigii, Pichia* spp., *H. uvarum* and *S. cerevisiae* were isolated at numbers greater than 1.0E+04 CFU/100 ml/berry. Although the numbers for *Pichia* spp. (in September 1999) were highly variable, *Pichia* spp. were represented at the highest average of 2.0E+05 CFU/100 ml/berry. *S. cerevisiae* was isolated only in October 1999 and this from leaves and overripe berries.

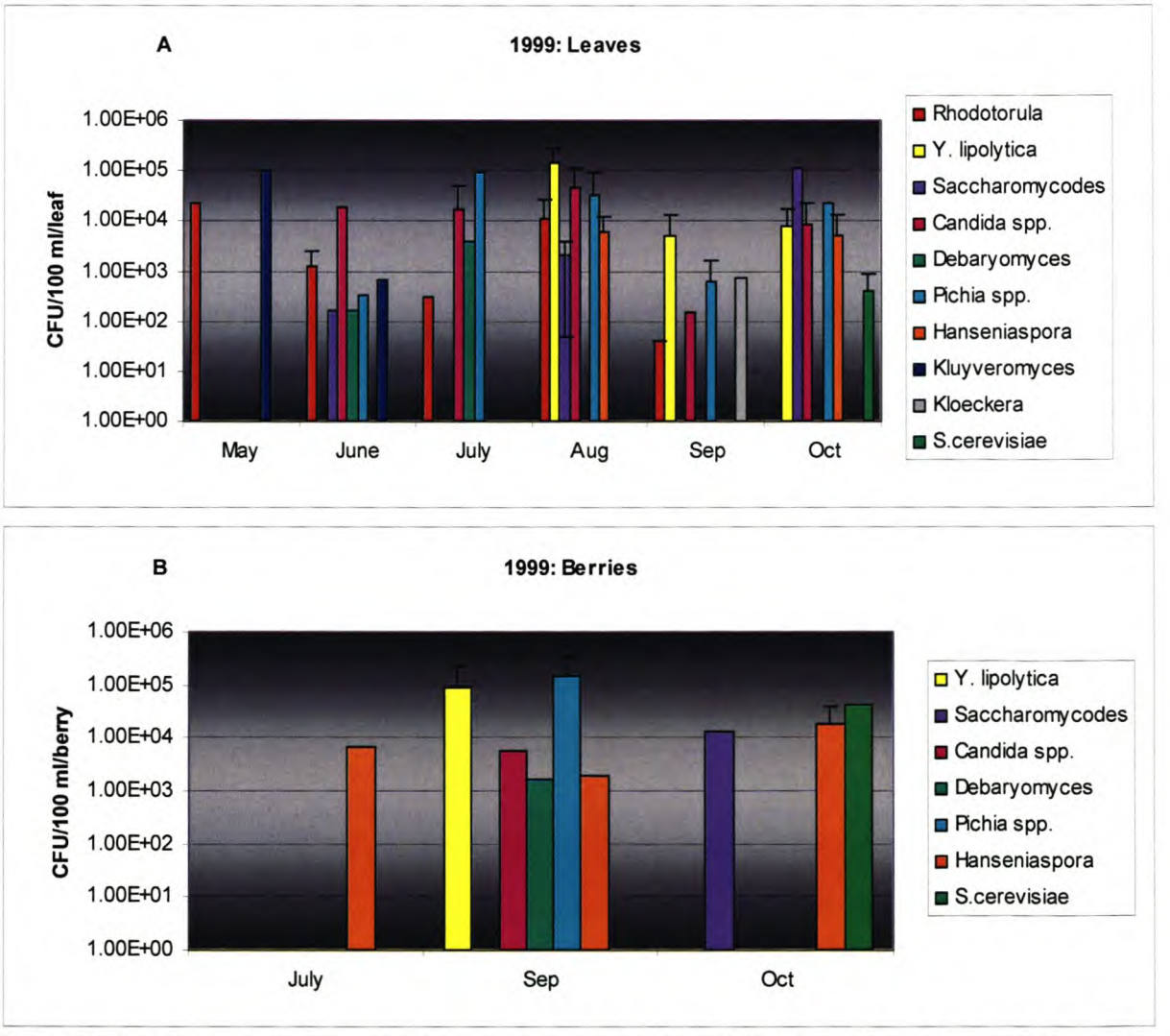

Fig. 3.3 Yeasts isolated from leaves (A) and berries (B) in 1999. *Saccharomycodes, Debaryomyces, Kluyveromyces, Hanseniaspora* and *Kloeckera* represent *Saccharomycodes ludwigii, Debaryomyces hansenii, Kluyveromyces lactis, Hanseniaspora uvarum and Kloeckera apiculata*, respectively.

3.3.1.1.1 *Candida* and *Pichia* spp.

The isolated *Candida* spp. consisted of *Candida parapsilosis*, *Candida lambica* and *Candida pulcherrima* and these mostly came from the leaves (Fig. 3.4A). *C. pulcherrima* (anamorf of *Metchnikowia pulcherrima*), *C. parapsilosis* and *C. lambica* were consistently isolated from the leaves for two or more months during the season. Although with some variation, *C. pulcherrima* was isolated at the highest average number of 7.0E+04 CFU/100 ml/leaf. *C. lambica* was the only *Candida* spp. isolated from the berries, albeit only once at an average number of 8.0E+03 CFU/100 ml/berry.

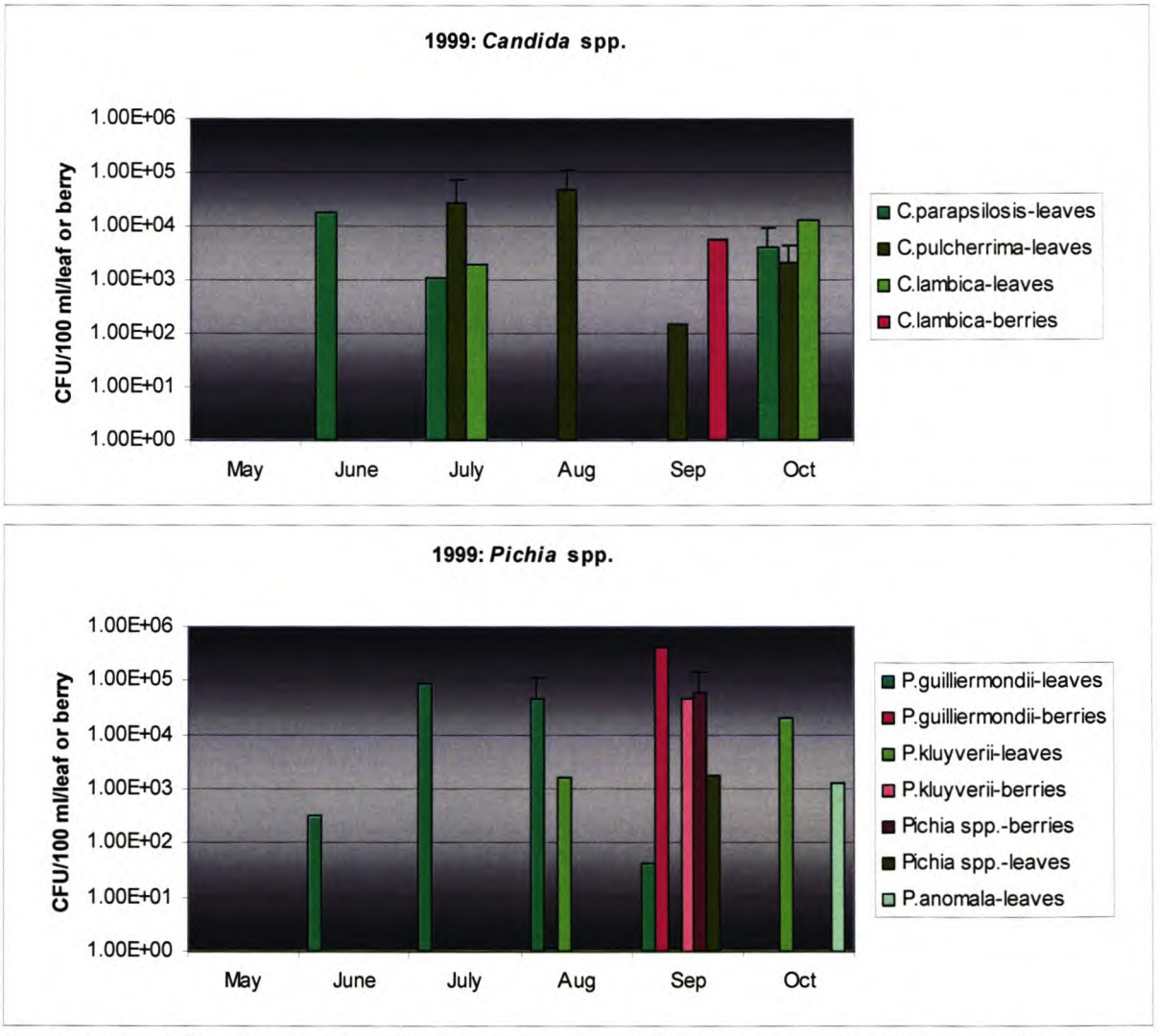

Fig. 3.4 The different *Candida* (A) and *Pichia* spp. (B) isolated from the leaves and berries in 1999.

Pichia spp. were represented by *Pichia guilliermondii, Pichia kluyverii, Pichia anomala* and other *Pichia* spp. that could not be characterised to species level. *P. guilliermondii* and *P. kluyverii* were consistently isolated from leaves over two or more months (Fig. 3.4B). *Pichia* spp. were not consistently isolated from berries in 1999. The highest counts for isolates from both leaves and berries were detected for

1999. The highest counts for isolates from both leaves and berries were detected for *P. guilliermondii* at 9.0E+04 CFU/100 ml/leaf and 6.0E+05 CFU/100 ml/berry, respectively. Evidently from the charts in Fig. 3.4, both *Candida* and *Pichia* spp. were isolated from berries during September 1999. From the leaves, however, isolates of both species were spread throughout the entire season.

From the time that they were seedlings, the grapevines used in this study were reared in a glasshouse environment and were never exposed to normal physical conditions in the vineyard. However, normal vineyard practices, e.g. spraying of fungicides and pruning, were carried out. There is nonetheless a good chance that the yeast populations on these grapevines would differ from the yeast populations on conventional grapevines.

3.3.1.2 Inoculation of GMY1 in 2000

3.3.1.2.1 Wild yeasts

From June until November 2000, sampling was done for 19 weeks and one GM yeast, GMY1, was inoculated in late August. A total of 338 yeast isolates representing 13 genera were isolated from the leaves and berries. In June, no berries were sampled and in November no leaves were sampled.

In the control block, *Rhodotorula, Y. lipolytica, Pichia* spp., *Cryptococcus*, *Aureobasidium, Candida* spp. and *S. cerevisiae* were consistently isolated from the leaves (Fig. 3.5A) and *Y. lipolytica, Pichia* spp., *Candida* spp. and *Aureobasidium* from the berries for either two or more months (Fig. 3.6A). *Rhodotorula, Y. lipolytica, Pichia* spp. and *Hanseniaspora* were the only yeasts that were isolated at numbers higher than 1.0E+03 CFU/100 ml/leaf from the leaves, while there was a tendency for the collective *Pichia* spp. to be present at the highest numbers (Fig. 3.5A). *Aureobasidium* was isolated from berries and the highest concentration of this fungus was 1.0E+04 CFU/100 ml/berry (Fig. 3.6A). The only other yeast that could be detected at numbers more than 1.0E+03 CFU/100 ml/berry, was the *Pichia* spp.

S. cerevisiae was also isolated from both the leaves and berries from this block, although in low numbers. This low number of wild *S. cerevisiae* isolates confirmed the general consensus that *S. cerevisiae* is not readily isolated from grapes (Fleet, 1993).

Comparing the yeast isolated in 1999 to that of the control block in 2000, it is evident that yeasts isolated from the leaves were spread out through the season. It is interesting that in general, yeasts in 1999 were isolated at higher average numbers than in 2000. With the exception of a few genera, e.g. *Aureobasidium* and *Cryptococcus,* the same yeasts were detected each year on both the leaves and berries. Compared to leaves, isolates from berries were not equally prevalent and it has already been partly discussed. It was also considerably more difficult to isolate yeasts from berries due to the inherent physical structure of grapes.

In block (VIN13), *Rhodotorula, Y. lipolytica,* and *Pichia* spp. were the yeasts isolated most often (for two months or more) from the leaves, at average counts of

more than 1.0E+03 CFU/100 ml/leaf (Fig. 3.5B). Despite some variation, *Pichia* spp. were isolated at the highest average of 9.0E+03 CFU/100 ml/leaf. Yeast numbers from the berries were considerably less than from the leaves. Only *Candida* spp. were detected consistently (for four months) but numbers were variable (Fig. 3.6B). *Candida* spp. were the wild yeasts to be detected at the highest concentration of 4.0E+03 CFU/100 ml/berry.

In block (GMY1), *Y. lipolytica* and *Pichia* spp. were isolated most often from the leaves (Fig. 3.5C). The *Pichia* spp. were the wild yeasts detected at the highest concentration of 2.0E+04 CFU/100 ml/leaf. Interestingly, there was a tendency for the *Pichia* spp. to be isolated at the highest average concentration on the leaves of all the treated blocks in 2000, except for block (VIN13+GMY1). No persistent isolates present at numbers higher than 1.0E+03 CFU/100 ml/berry could be detected on the berries from block (GMY1) (Fig. 3.6C). Although in lower numbers compared to the leaves, *Y. lipolytica, Pichia* and *Candida* spp. were frequently isolated from the berries for two months or more. *Candida* spp. were detected at the highest average of 3.0E+02 CFU/100 ml/berry. From all the treated blocks, wild *S. cerevisiae* was only isolated from grapevines in this block.

In block (VIN13+GMY1), most of the yeasts isolated from the leaves were often detected. Of these yeasts, *Rhodotorula, Pichia* spp. and *Aureobasidium* were present in numbers higher than 1.0E+03 CFU/100 ml/leaf (Fig. 3.5D). In this case, *Cryptococcus* was detected at the highest number of 7.0E+04 CFU/100 ml/leaf. From the berries in this block, most yeasts were also persistently detected, although again in much lower numbers than on leaves. Although there was some variation, only the *Pichia* spp. were present at a concentration higher than 1.0E+03 CFU/100 ml/berry (Fig. 3.6D).

Rhodotorula, *Y. lipolytica, Pichia* spp., *Candida* spp., *Hanseniaspora, Aureobasidium, Cryptococcus,* and *Debaryomyces* were present on the grapevines from all three blocks. These yeasts were also detected in the control block. There was therefore no significant change in the variety of wild yeasts detected following the introduction of VIN13 and GMY1 in the treated blocks. Overall in 2000, the greatest variety of wild yeasts was isolated during September. Considering that the introduction of the inoculated yeasts into the normal wild yeast populations occurred in August 2000, the prevalence of native yeasts was not affected.

Overall, species of *Candida* and *Pichi*a were among the dominant yeasts present on the leaves and berries. It is known that, as fruit deteriorates, yeasts that are capable of utilising a broader spectrum of carbon and energy sources, such as *Pichia* and *Candida* spp., become more numerous on the surface of fruit (Morais *et al.*, 1995; Abranches *et al.*, 2000).

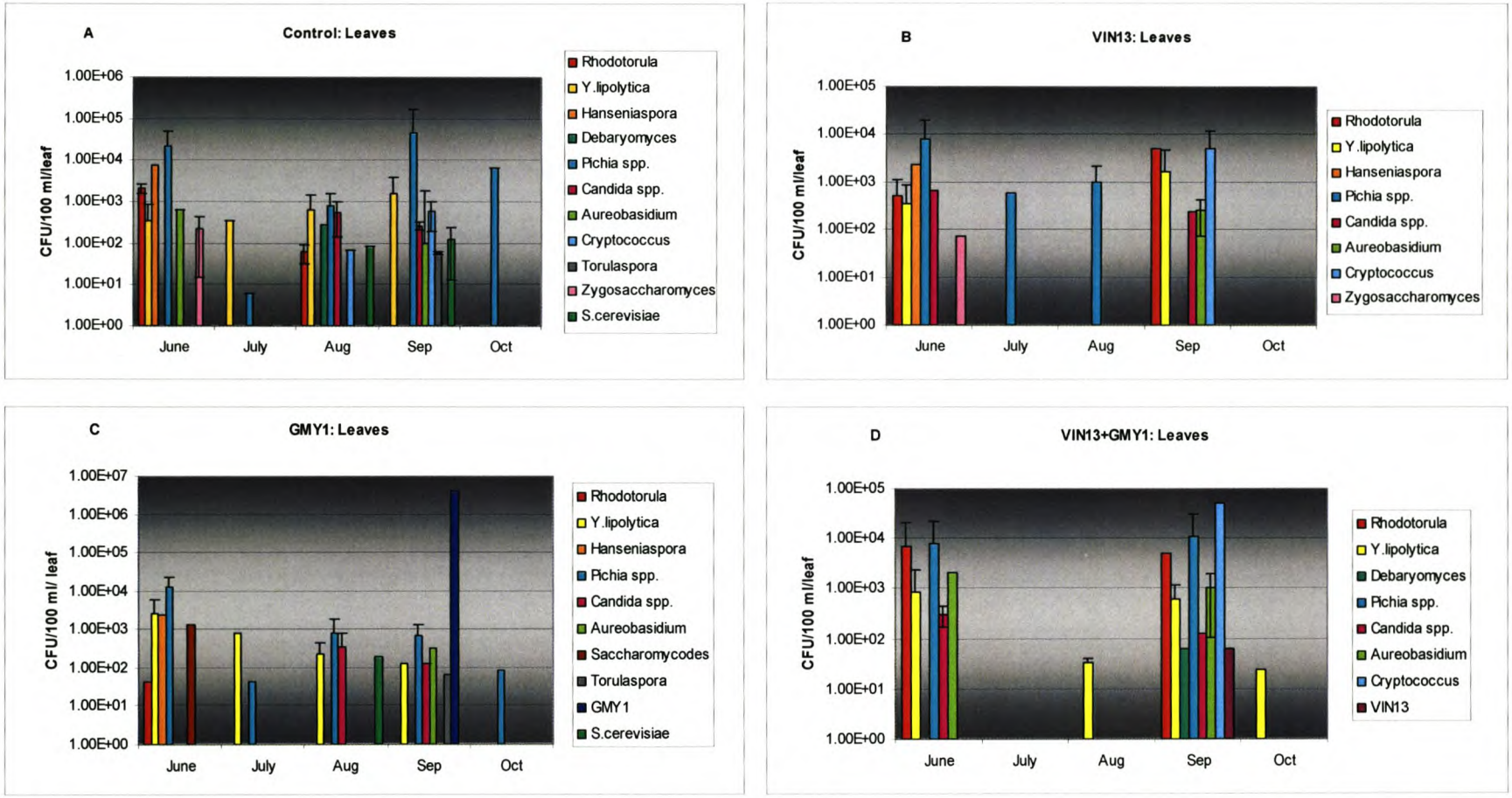

Fig. 3.5 Yeasts isolated from the leaves in all blocks in 2000. VIN13 and GMY1 were inoculated in all blocks in August 2000. In (A) and (D) *Debaryomyces* represents *D. hansenii* and *D. mycophilus*, respectively. *Torulaspora* represents *T. delbrueckii.*

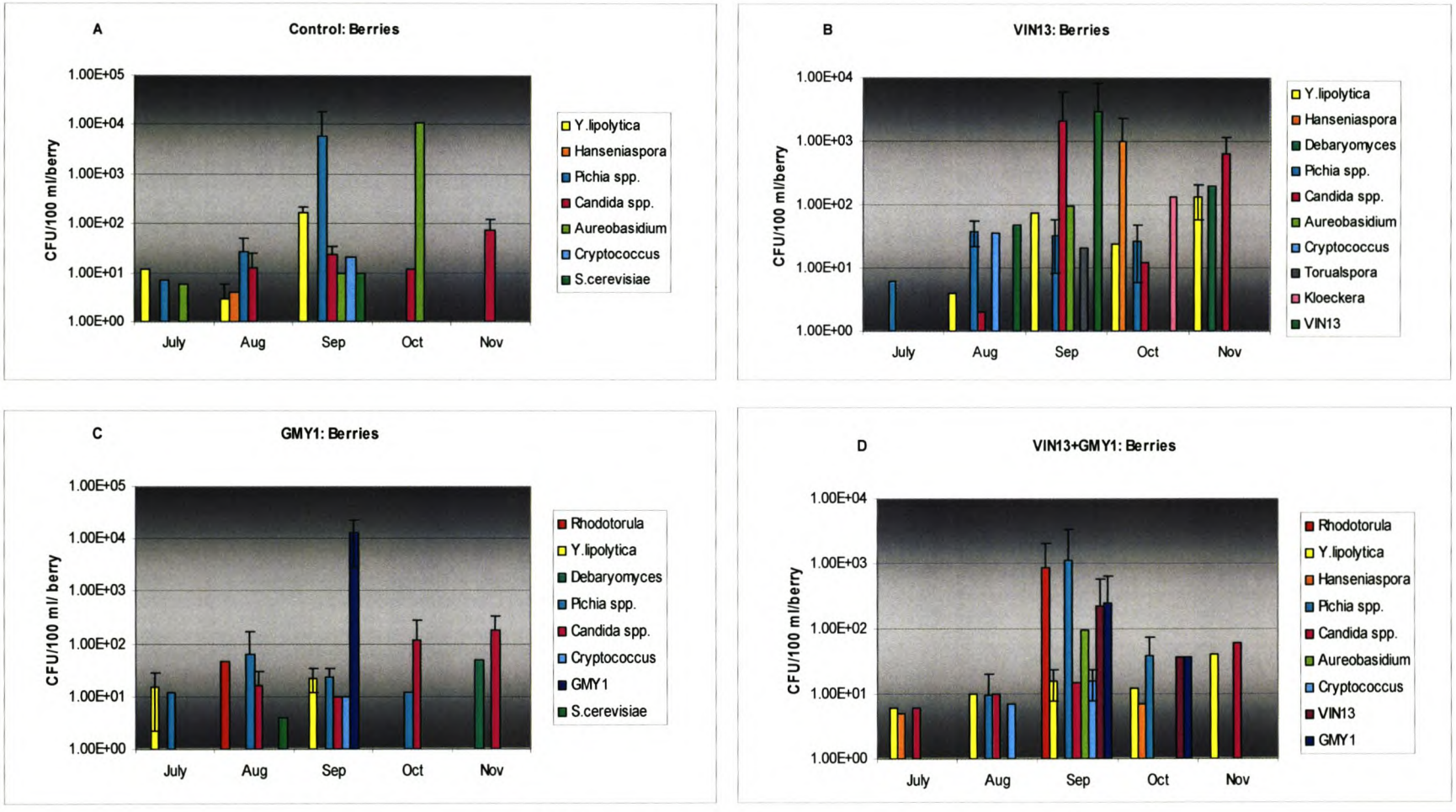

Fig. 3.6 Yeasts isolated from berries in all blocks in 2000. In (B) and (C) *Debaryomyces* represents *D. hansenii*.

3.3.1.2.2 *Candida* and *Pichia* spp.

Candida spp. that were isolated in 2000 included *C. pulcherrima, Candida musae, Candida maltosa, C. parapsilosis, Candida pseudointermedia, Candida lyxosophila, Candida austromarina, Candida tropicalis* and other *Candida* spp. not characterised to species level. In the control block, *C. pulcherrima* was the only *Candida* spp. that was isolated from the leaves in more than one month (two months) with the highest average of 5.00E+02 CFU/100 ml/leaf (Fig. 3.7A). From the berries, the most frequently isolated species were *C. parapsilosis* and *C. lyxosophila*. Although not always isolated, *C. musae* was detected in some samples, with a highest count of 1.00E+02 CFU/100 ml/berry (Fig. 3.7A). Interestingly, a variety of four different *Candida* spp. were isolated during November 2000 when the berries were very ripe and starting to rot.

Pichia spp. included *P. guilliermondii, P. anomala, Pichia pastoris* and *Pichia* spp. not characterised up to species level. *P. guilliermondii* and *P. anomala* were the *Pichia* spp. consistently isolated from both the leaves and berries in the control block for at least three months during the season (Fig. 3.7B). *P. anomala* was detected with highest counts of 1.00E+05 CFU/100 ml/leaf and 2.00E+04 CFU/100 ml/berry, respectively. There was, however, some variation in the detected *P. anomala* from the leaves during September 2000. Overall, *Pichia* spp. were more consistently isolated throughout the season, while most *Candida* spp. were found during August and September 2000. The highest average number of yeast isolates from grapevines was always higher for the leaves than the berries.

A comparison of *Candida* and *Pichia* spp. isolates from 1999 and 2000 (the control block) revealed that *C. pulcherrima* and *P. guilliermondii* were the most prevalent isolates from leaves for both years. However, isolates from the berries differed between the two years. For instance, in 1999, *Candida* and *Pichia* spp. isolates showed substantial fluctuations, while during the season of 2000, *C. parapsilosis, C. lyxosophila, P. guilliermondii* and *P. anomala* were persistently detected.

The isolated *Candida* spp. in block (VIN13) were not prevalent on leaves. *C. parapsilosis* was isolated at the highest average of 8.0E+02 CFU/100 ml/leaf (Fig. 3.8A). *Candida* spp. that were regularly isolated from the berries (over three months) were *C. austromarina* and *C. parapsilosis*, of which *C. austromarina* was isolated at the highest average of 7.0E+03 CFU.100 ml/berry (Fig. 3.8A). There was a greater variety of *Candida* spp. present on berries than on leaves. Considering the average numbers of all the *Candida* isolates throughout the season, it can be seen that roughly ¾ of the total *Candida* isolates were from berries (Fig. 3.8B). *P. guilliermondii* was consistently isolated from leaves and berries in block (VIN13) (Fig. 3.9A). Although in lower numbers, *P. anomala* was also well represented on berries and consistently isolated over three months. *P. guilliermondii* was isolated at the highest average numbers from leaves and berries. *Pichia* spp. isolates from the

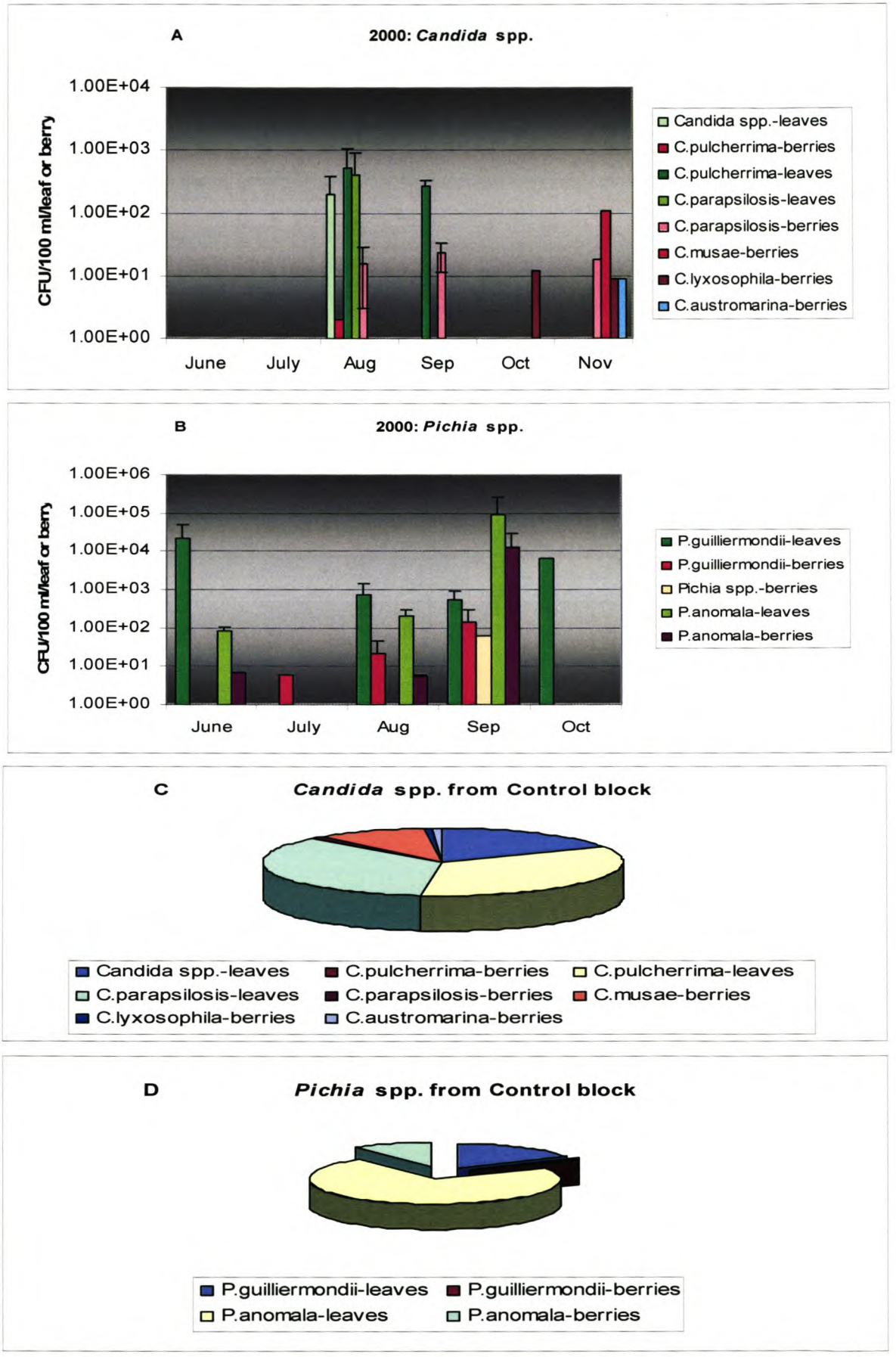

Fig. 3.7 The different *Candida* spp. (A) and *Pichia* spp. (B) isolated from the control block in 2000. (C) and (D) represent the average numbers of all *Candida* spp. and *Pichia* spp. isolates throughout the season.

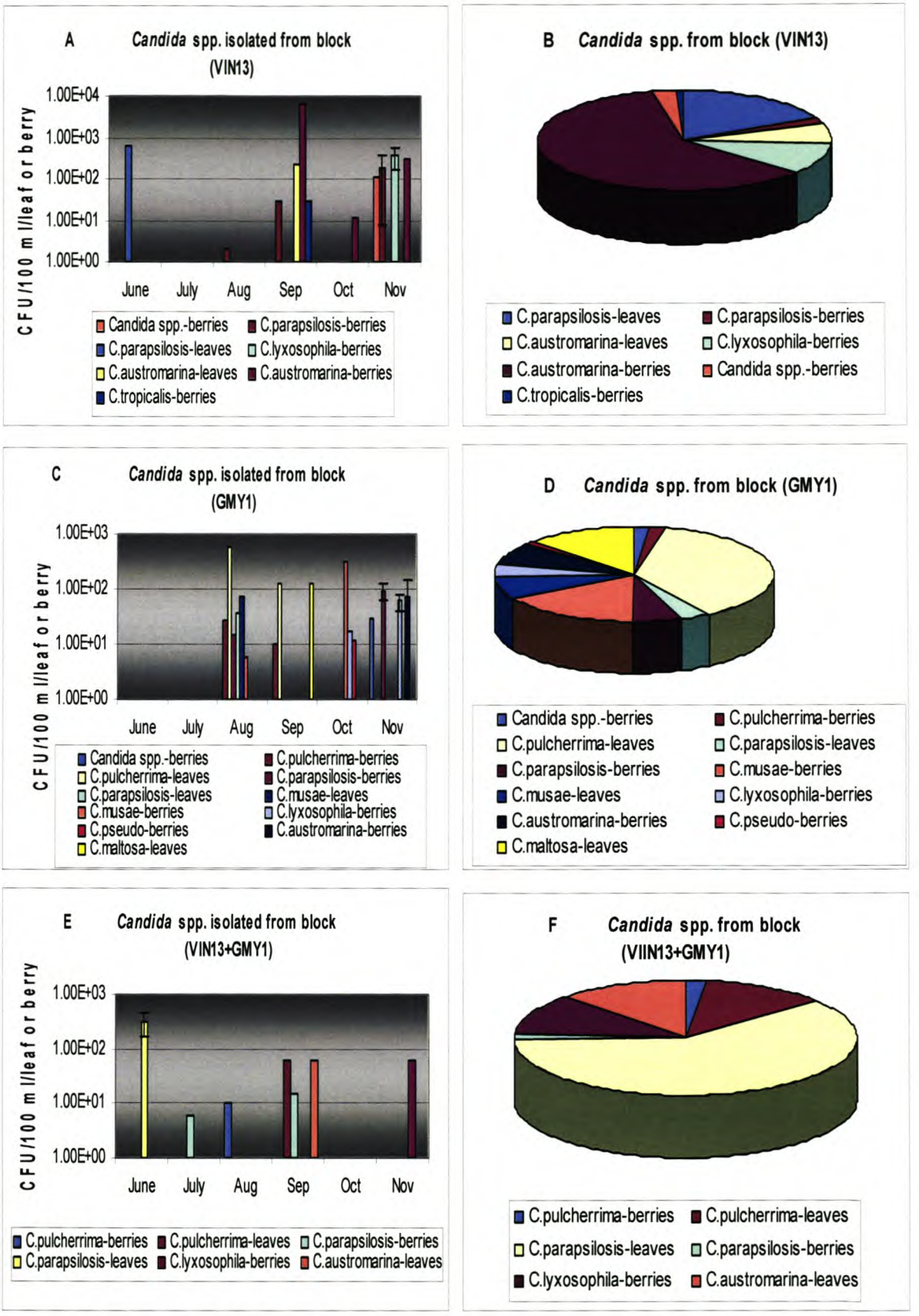

Fig. 3.8 The *Candida* spp. isolated from different treated blocks in 2000. (A) and (B) represent species isolated from block (VIN13), (C) and (D) from block (GMY1) and (E) and (F) from block (VIN13+GMY1). (B), (D) and (F) represent the average numbers of all *Candida* spp. isolates throughout the season. C. pseudo = *C. pseudointermedia*.

leaves represented the highest average number of yeasts throughout the season. A small part of the total isolated yeasts originated from the berries (Fig. 3.9B).

In block (GMY1), only *C. pulcherrima* was persistent on the leaves with a highest average count of 7.0E+02 CFU/100 ml/leaf (Fig. 3.8C). A greater variety of *Candida* spp. was isolated from berries over more than two months: *C. parapsilosis, C. pulcherrima, C. musae* and C. *lyxosophila*. *C. musae* had the highest average number of 5.0E+02 CFU/100 ml/berry. Roughly ¾ of all the *Candida* spp. isolates in the season were from leaves, although a greater variety of species was detected on the berries (Fig. 3.8D). *P. guilliermondii* and *P. anomala* were isolated most often from leaves in this block with *P. guilliermondii* at the highest average of 2.0E+04 CFU/100 ml/leaf. *P. guilliermondii* was also most prevalent on the berries with the highest average of 8.0E+01 CFU/100 ml/berry (Fig. 3.9C). On the whole, most of the *Pichia* spp. isolates came from leaves (Fig. 3.9D).

In block (VIN13+GMY1), *Candida* spp. were not frequently isolated from the leaves; no species was isolated over more than two months (Fig. 3.8E). *C. parapsilosis* was present at the highest average of 5.0E+02 CFU/100 ml/leaf at one sampling interval. From the berries, only *C. parapsilosis* was isolated more than once, while *C. lyxosophila* was present at the highest average of 8.0E+01 CFU/100 ml/berry. Roughly more than ¾ of the total *Candida* spp. isolates originated from leaves (Fig. 3.8F). In this block, *P. gulliermondii* was again present in relative high numbers on both the leaves and berries, although there was some variation in the prevalence of *P. guilliermondii* on leaves. *P. anomala* was also consistently isolated from berries (Fig. 3.9E). Again, by far the most *Pichia* spp. isolates originated from leaves (Fig. 3.9F).

When all the *Candida* spp. isolated from the three differently treated blocks are taken into account, it is evident that yeasts were not consistently isolated from leaves. Very few species were detected on more than one sampling date. *Candida* spp. were more readily isolated from the berries. The greatest variety of yeasts was present on the berries in block (GMY1). Overall, *C. parapsilosis* (all three blocks) and *C. lyxosophila* (two blocks) were isolated most often from the berries in the glasshouse. While there was a tendency for more *Candida* spp. to be isolated from August 2000 onwards (Fig. 3.8A, C and E), the isolation of *Pichia* spp. was more spread out through the season from June to October 2000 (Fig. 3.9A, C and E). In comparison, the variety and occurrence of *Candida* spp. between the inoculated blocks (Fig. 3.8B, D and F) and the control block (Fig. 3.7C) was variable and inconsistent. It is therefore concluded that the introduction of the GM yeasts had no significant effect on the occurrence of *Candida* spp. *P. guilliermondii* was consistently isolated from both the leaves and berries from all three treated blocks. *P. anomala* was also present often in two of the three blocks. From the charts, it is clear that the occurrence of *Pichia* spp. was remarkably similar in all three inoculated blocks (Fig. 3.9B, D and F). The occurrence of these species in the control block, however, was different compared to the rest of the blocks. *P. anomala*, which was

detected on leaves and berries, contributed more to the total *Pichia* spp. isolated than *P. guilliermondii*, which was detected only on leaves (Fig. 3.7D). It is therefore

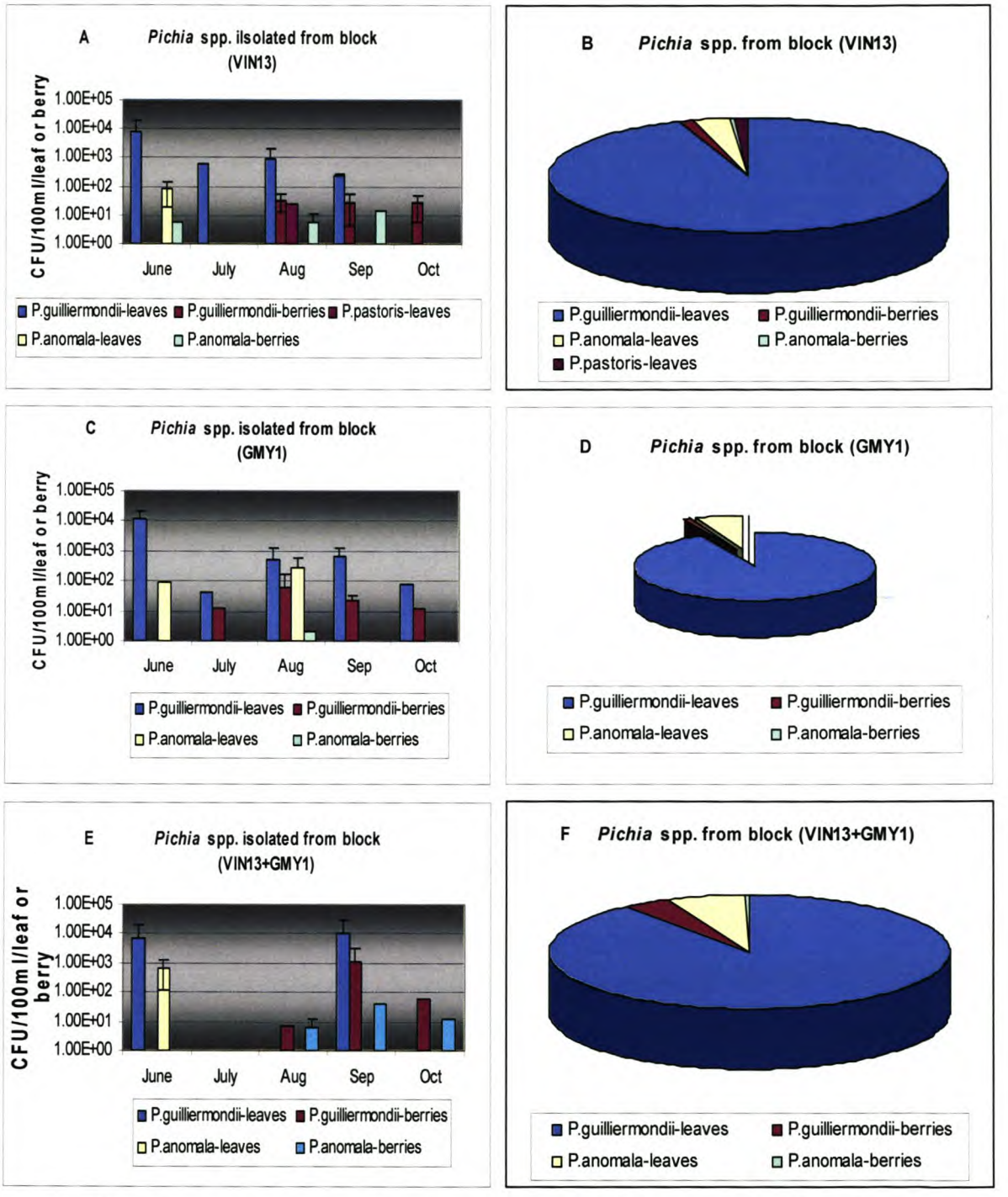

Fig. 3.9 *Pichia* spp. isolated from the different treated blocks in 2000. (A) and (B) represent species isolated from block (VIN13); (C) and (D) from block (GMY1) and (E) and (F) from block (VIN13+GMY1). (B), (D) and (F) represent the average numbers of all *Pichia* spp. isolates throughout the season.

possible that the intentional additions of the test strains had an effect on the relative abundance of the naturally-occurring *Pichia* spp. However, and more importantly, there was no difference in the effect of the modified and the wild *Saccharomyces* on the occurrence of *Pichia* spp.

3.3.1.2.3 Detection of inoculated yeasts: VIN13 and GMY1

In block (VIN13), VIN13 was only isolated from the berries; specifically once during August and several times during September 2000 (Fig. 3.6B). Although there was some variation in the levels of VIN13 during September 2000, this yeast was isolated at a relatively high concentration of 5.0E+03 CFU/100 ml/berry. Wild *S. cerevisiae* was distinguished from VIN13 by the PCR technique described in section 3.2.8 (results not shown).

In block (GMY1), GMY1 was isolated from leaves on one sampling date (September 2000) at a high count of 6.0E+06 CFU/100 ml/leaf (Fig. 3.5C). GMY1 was isolated more often from berries in September 2000 and also at a relatively high average of 2.0E+04 CFU/100 ml/berry (Fig. 3.6C).

In block (VIN13+GMY1), both VIN13 and GMY1 were rare isolates from leaves (VIN13 once only in September 2000) (Fig. 3.5D) and highly variable from berries appearing only in September and October 2000 (Fig. 3.6D). Overall, these yeasts were isolated together at roughly equal numbers and therefore approximately in the same ratio at which they were inoculated (1:1). Both these yeasts were never found at average numbers of more than 3.0E+02 CFU/100 ml/berry.

3.3.1.3 Inoculation of GMY1, GMY2 and GMY3 in 2001

3.3.1.3.1 Wild yeasts

In 2001, samples were collected on 11 occasions during the months of May, and August through October. Three GM yeasts, namely GMY1, GMY2 and GMY3 were inoculated onto the grapevines in late August. A total of 132 yeast isolates representing 9 genera were isolated from the leaves and berries. In comparison to 2000, the grapevines carried much less fruit; it was, by now, the third year that the grapevines were kept in pots and therefore they yielded a smaller crop. Due to time constraints, the emphasis in 2001 was primarily on detection of GM yeasts on the leaves and berries, although some sampling of wild yeasts was performed during the early growth season (May) and from August through October. *Rhodotorula, Pichia* spp., *Aureobasidium* and *Candida* spp. were isolated from the leaves in all four blocks (Fig. 3.10A to 3.10D). In the control block, *Pichia* spp. and *Saccharomyces* were isolated with counts higher than 1.0E+03 CFU/100 ml/leaf from leaves (Fig. 3.10A). *S. cerevisiae* was also isolated at a low count from berries in this block.

In block (VIN13), *Rhodotorula* was detected from the leaves at the highest concentration (Fig. 3.10B). VIN13 was the only yeast isolated from berries.

In block (GMY1+GMY2), a bigger variety of wild yeasts was present on the leaves. Among this *Pichia* spp. were, although detected in variable numbers, present at counts of more than 1.0E+03 CFU/100 ml/leaf (Fig. 3.10C).

In block (GMY1+GMY2+GMY3), *Candida* and *Pichia* spp. were isolated from leaves at more than one sampling event (Fig. 3.10 D). *Pichia* spp. and *Y. lipolytica*

were present at numbers higher than 1.0E+03 CFU/100 ml/leaf. *Y. lipolytica, Candida* spp. and *Torulaspora* were isolated most often from berries.

Yeasts associated with winemaking represent 15 of the 100 yeast genera described in the latest edition of the *The Yeasts, A Taxonomic Study* (Kurtzman and Fell, 1998). These genera are represented by *Brettanomyces* and its sexual equivalent, *Dekkera; Candida, Cryptococcus, Debaryomyces, Hanseniaspora* and its asexual equivalent *Kloeckera*; *Kluyveromyces, Metschnikowia, Pichia, Rhodotorula*, *Saccharomyces, Saccharomycodes, Schizosaccharomyces* and *Zygosaccharomyces* (Pretorius *et al.*, 1999). Roughly 50-75% of the total yeast flora recovered from grape berries, consist of the low alcohol-tolerant species of *Kloeckera* and its teleomorph *Hanseniaspora* (e.g. *K. apiculata* and *H. uvarum*). Less prevalent on the grapes are species of *Candida* (e.g. *C. pulcherrima* and *C. stellata*), *Pichia* (as well as those species previously assigned to the genus *Hansenula*), *Rhodotorula, Kluyveromyces* and *Cryptococcus*. It is estimated that the total yeast population present on a healthy, grape berry is 10^3-10^5 CFU/ml (Fleet, 1993). Although in low numbers, the wild yeasts isolated from the grapevines each year for this study were typical wine-associated yeasts. Yeasts isolated from grapes were however not detected in the same numbers as expected, while yeasts normally expected to be less prevalent on grapes (i.e. *Candida*, *Pichia, Rhodotorula* and *Cryptococcus*) were isolated most frequently from the grapes in the glasshouse. *Kloeckera* and *Hanseniaspora*, which are reportedly the most prevalent yeasts on grapes (Fleet *et al.*, 2002) were seldom detected.

An interesting observation was that more yeasts were isolated from the combination of leaves at the top and bottom of grapevines than from combined leaves on the sunny and shady sides of grapevines (results not shown). This could possibly be due to carriage by small insects that entered the glasshouse through the roof windows, and preferred to dwell on the top part of the grapevines. It is known that insects play an important role in spreading yeasts in the vineyard.

Evidence based on direct isolation procedures without any enrichment clearly indicates the extreme difficulty with which *S. cerevisiae* is isolated from habitats, such as vineyard soil and the surfaces of ripe grapes (<10 CFU/cm^2 of fruit surface or gram of soil) or any other sweet fruit (Martini, 1993). Overall, it was our experience that *S. cerevisiae* was not often isolated. In 1999, this yeast was only detected for one month on the grapevines (Fig. 3.3).

The microflora of grapes is highly variable. Several factors can affect the diversity of yeast flora isolated in ecological studies, namely climate, grape variety, soil quality, soil fertilisation, irrigation and viticultural practices, physical damage caused by moulds, insects and birds, fungicides (Pretorius *et al.,* 1999), geographic location, age of the vineyard, grape maturity and the isolation techniques used (Martini *et al.,* 1980; Rosini *et al.*, 1982). One possibility for a future study of this nature would be to use an older and different variety of grapevine.

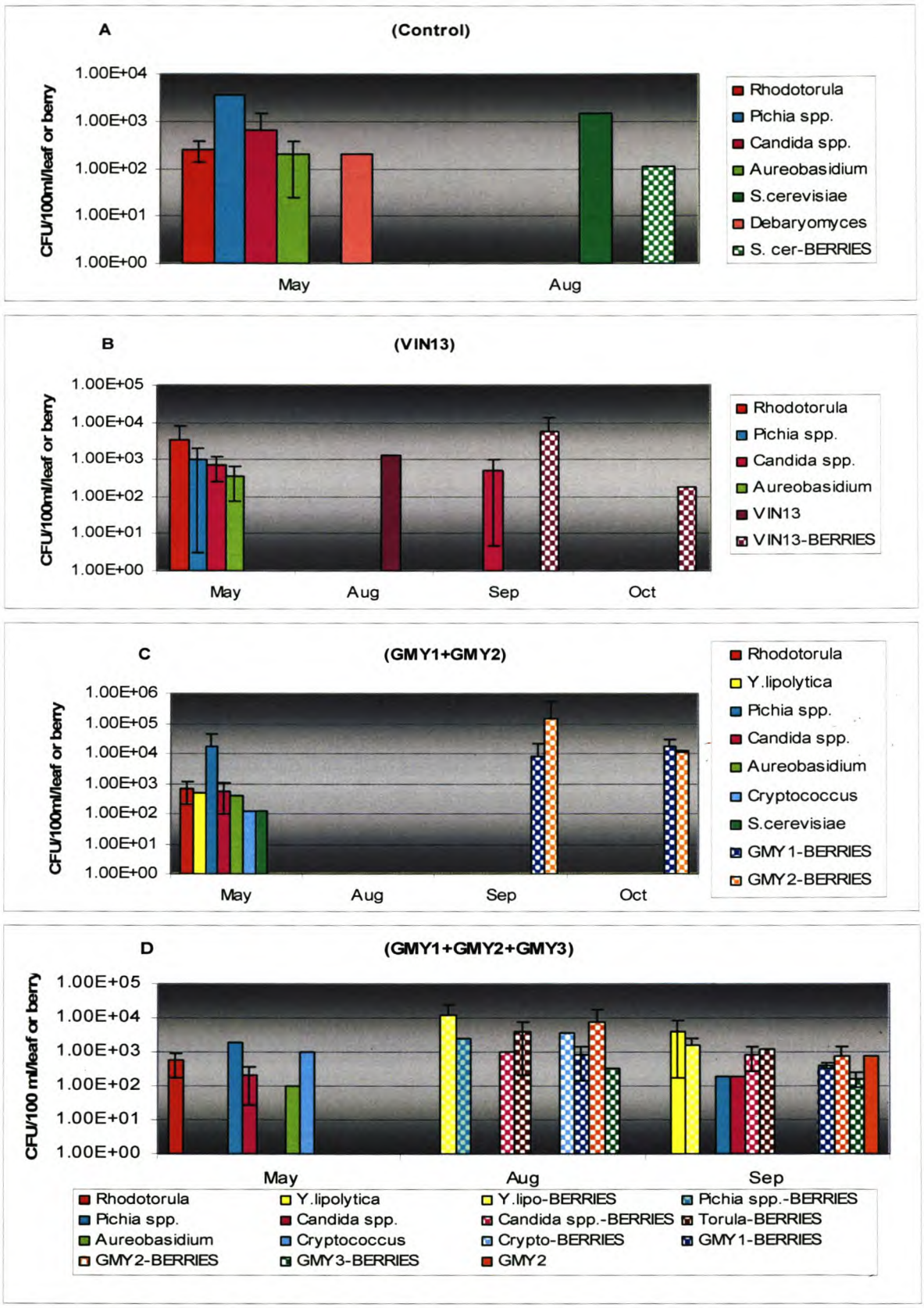

Fig. 3.10 Yeast isolates from leaves and berries in the control block (A), VIN13 (B), (GMY1+GMY2) (C) and (GMY1+GMY2+GMY3) (D) during 2001. *Debaryomyces* in (A) represents *D. costelli* and *Torulaspora* in (D), *T. delbrueckii*. *Cryptococcus* in (C) represents *C. saitoi* and in (D), *C. curvatus* (leaves) and *C. laurentii* (berries). S. cer-BERRIES, Y. lipo-BERRIES, Crypto-BERRIES and Torula-BERRIES represent *S. cerevisiae, Y. lipolytica, Cryptococcus* and *Torulaspora* isolated from the berries. Where yeasts were isolated from the leaves, only the yeast name is indicated.

An important aspect of this project was the identification of all isolated yeasts. The conventional method for yeast identification involves a complex, laborious and time-consuming process that can require some 60-90 tests (Deák, 1995). These tests can also possibly be influenced by the culture medium and are therefore hard to interpret and unsuitable for precise yeast identification (Deák, 1993). Classical morphological and biochemical methods of identification were initially coupled with Clamped Homogenous Electric Fields (CHEF) gel electrophoresis. The latter method of identification was successful to a limited extent, since this technique is not yet optimised and refined for wild-type yeasts. In the end, PCR with the D1/D2 primers from the 26S rRNA gene was used as the method of yeast identification (see section 3.2.7). Although relatively expensive, this was the most effective and reliable way of yeast identification.

3.3.1.3.2 *Candida* and *Pichia* spp.

Candida and *Pichia* spp. were often isolated from grapevines in 2001. Collectively, *Candida* spp. consisted of *C. pulcherrima, Candida norvegica, C. maltosa, C. parapsilosis, Candida galli, C. lyxosophila, Candida quercitrusa, Candida oleophila, Candida zeylanoides, Candida fermentati, C. austromarina* and isolates that could not be characterised to the species level. *C. pulcherrima* and *C. parapsilosis* were isolated from the leaves in all four blocks, while the rest of the species varied between the blocks (Fig. 3.11A to 3.11D). With the exception of *C. maltosa*, which was detected at counts higher than 1.0E+03 CFU/100 ml/leaf in the control block, all *Candida* spp. were isolated at between 1.0E+02 and 1.0E+03 CFU/100 ml/leaf or berry (Fig. 3.11A). In block (GMY1+GMY2+GMY3) isolates from the berries were frequent and different *Candida* spp. were detected (Fig. 3.11D). With the exception of *C. quercitrusa, C. galli* and *C. oleophila* were isolated from the berries.

The collective *Pichia* spp. isolated consisted of *P. guilliermondii, P. anomala, Pichia xylosa* and *Pichia* spp. that could not be characterised up to species level. *P. anomala* was isolated from the leaves in all four blocks at concentrations higher than 1.0E+03 CFU/100 ml/leaf (Fig. 3.12A to 3.12D), while *P. guilliermondii* was also persistent (Fig. 3.12B to 3.12D). The latter yeast was also isolated at a relatively high concentration (more than 1.0E+03 CFU/100 ml/berry) from the berries in block (GMY1+GMY2+GMY3) (Fig. 3.12D).

Since fewer wild yeasts were isolated during 2001, statistical comparison between the yeasts of all three years of isolation can not be made. Nevertheless, there was a strong trend showing that *C. pulcherrima, C. parapsilosis, P. guilliermondii* and *P. anomala* were the dominant *Candida* and *Pichia* spp. that were isolated from the grapevines over this period.

3.3.1.3.3 Detection of inoculated yeasts: VIN13, GMY1, GMY2, and GMY3

VIN13 was isolated from leaves (once in August 2001) and berries in block (VIN13) (Fig. 3.10B). VIN13 was detected at variable numbers on the berries during September 2001, where it peaked at 8.0E+03 CFU/100 ml/berry, after which its numbers declined. During 2000 and 2001, VIN13 was not prevalent on the leaves, but was most frequently isolated from berries during September of each year (also see Fig. 3.5B and 3.6B). After September, the yeast declined in number (2001) or could not be isolated at all (2000).

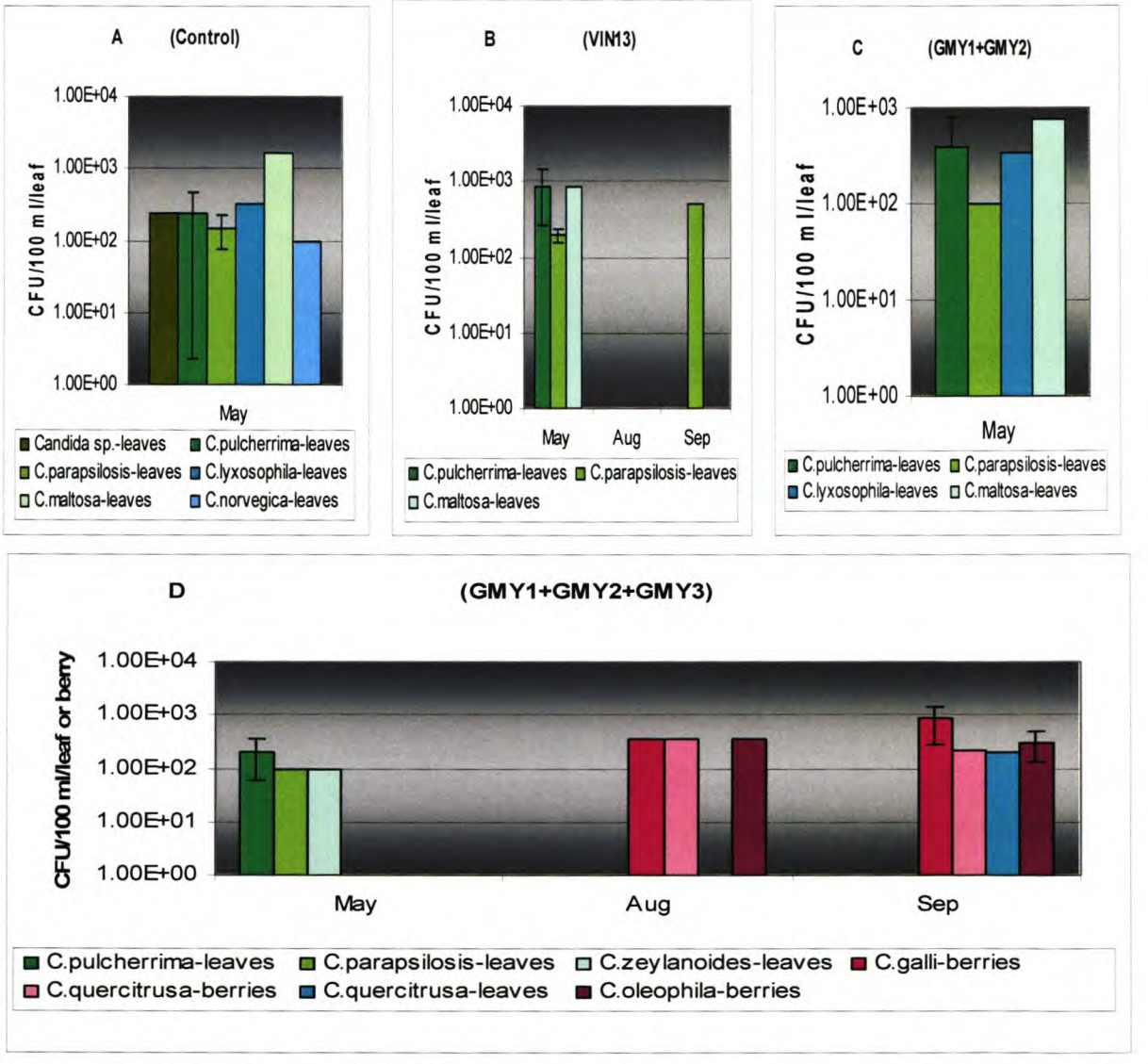

Fig. 3.11 The *Candida* spp. isolated from the different treated blocks in 2001. *Candida* spp. were only isolated from the leaves in the control block (A), block (VIN13) (B) and block (GMY1+GMY2) (C). *Candida* spp. isolated from the leaves and berries in block (GMY1+GMY2+GMY3) is represented by (D).

GMY1 and GMY2 were isolated from berries in block (GMY1+GMY2) in relatively high numbers (more than 1.0E+03 CFU/100 ml/berry) (Fig. 3.10C). In the first month after inoculation, the detected concentrations of GMY1 and GMY2 were highly variable. Overall, GMY1 was detected at an average lower level than that of GMY2.

In October 2001, GMY1 was again detected but at a higher level and GMY2 numbers had declined. In fact, during this month GMY1 and GMY2 were isolated in nearly equal numbers: 1.69E+04 and 1.14E+04 CFU/100 ml/berry, respectively.

Only GMY2 was isolated from leaves in block (GMY1+GMY2+GMY3) (Fig. 3.10D). Interestingly, the pattern of GM yeast detection on berries was GMY2>GMY1>GMY3 for August and September 2001. In the second month, the average numbers also declined. Except for the isolation of GMY2 in August 2001, which was also highly variable, no GM yeast could be detected at concentrations higher than 1.0E+03 CFU/100 ml/leaf or berry.

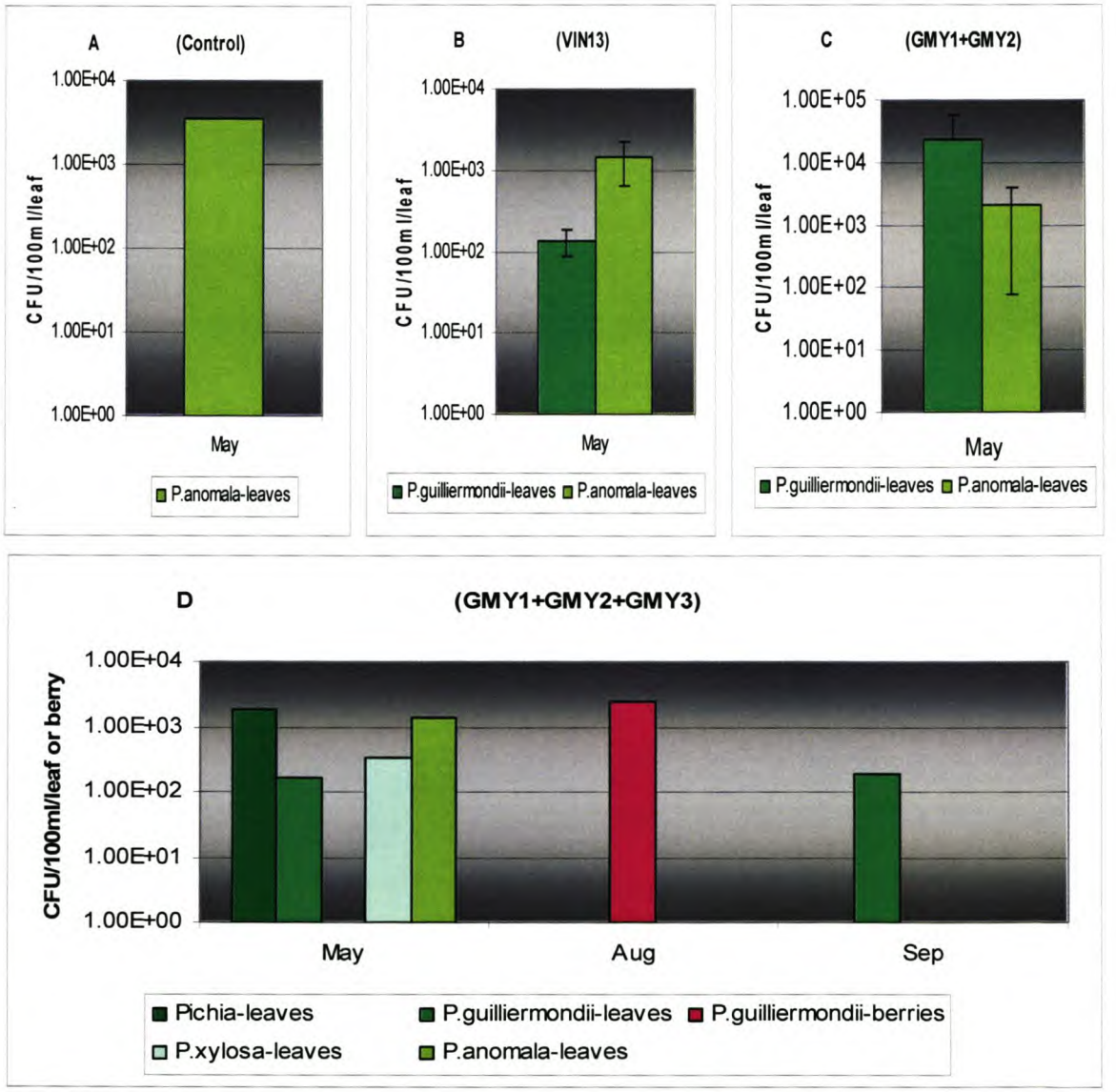

Fig. 3.12 *Pichia* spp. isolated from different treated blocks in 2001. (A), (B) and (C) represent species isolated from leaves in the control block, block (VIN13), and block (GMY1+GMY2), respectively. *Pichia* spp. from both the leaves and berries in block (GMY1+GMY2+GMY3) is shown in (D).

It may have been expected to isolate more inoculated yeasts from grapes as a result of the dosage effect. However, the results of culturable cell counts demonstrate that the detection of GM yeasts on the surface of grapes was limited. In

2000, GMY1 on leaves (>1.0E+06 CFU/100 ml/leaf) (Fig. 3.5C) and berries (>1.0E+04 CFU/100 ml/berry) (Fig. 3.6C) was high relative compared to wild yeasts. In 2001, most GMY1 and GMY2 in block (GMY1+GMY2) were also present in numbers higher than 1.0E+04 CFU/100 ml/berry (Fig. 3.10C).

Filamentous fungal growth on plates in the laboratory interfered at times with yeast enrichments. Several fungal inhibitors were tested at different concentrations and it was found that Biphenyl (0.04%) dissolved in ethanol and sodium propionate at concentration (0.1 - 0.2%), inhibited fungi the best. It is possible that the fungus inhibitor could have had an effect on yeast growth, since plates without inhibitor had many fungi, bacteria and little yeast growth on them, while in many instances, plates with inhibitor had almost no growth. Overall, no fungal inhibitor was completely effective. Previously, enrichment with ethanol was used to isolate *S. cerevisiae* from vineyard grapes (Török *et al.*, 1996; Mortimer and Polsinelli, 1999). YM plates containing 12% ethanol were used to isolate *Saccharomyces* and also completely ruled out the problem with fungal contamination. However, the use of ethanol in plates may have inhibited the isolation of other grapevine-associated yeast species.

The method of yeast isolation that was used in this study is also routinely used by a research station in Wädenswil, Switzerland (personal communication: Petra Hoffman-Boller, Analytical and Technological Department). From our experience it was evident that there can be a notable difference in dynamics between yeasts over time. Several attempts were made to improve yeast isolations, e.g. by using detergents (10%SDS, Tween 80, Triton X-100) and experimenting with the time of washing. Even the sonication of berries for several time intervals did not reveal any more yeasts than were normally isolated. The specific structure of the grape berry could be the reason why the yeasts were difficult to remove. The surface of a plant is a feature that often affects results in studies of microbial ecology. Lesions and organic secretions on plant surfaces may firmly entrap micro-colonies in small and deep channels and/or in waxy and mucous materials (Martini *et al.*, 1996). More aggressive and cell-dislodging procedures could have led to better yeast isolations. However, it cannot be excluded that the microflora on the grapes originating from a secluded glasshouse environment could have a far less diverse nature of yeast dynamics.

The use of sterile swabs at strategic places, e.g. underneath grapevines and on the plastic sheets on which the grapevines were positioned, demonstrated that GM yeasts were absent at other locations in the glasshouse.

3.3.2 INFLUENCE OF FUNGICIDE ON THE YEASTS

The grapevines in the glasshouse were subjected to normal viticultural practices, with a fungicide being applied to the grapevines. Ridomil, was sprayed (in July each year) at a dosage of 0.15% on the grapevines and could be seen as a thick, white precipitate in the washing buffer after each isolation, especially in the first few weeks after application. Fewer yeasts were isolated during and soon after the week of

application in 2000. It was suspected that Ridomil influenced yeast survival and therefore a few yeast strains were tested for their sensitivity to the fungicide. No effect on yeast viability was observed after three days of incubation with the fungicide and only slight decreases in the cell viability of the treated yeasts could be detected after seven days (results not shown). However, significant decreases in cell viability were observed with VIN13 and the GM yeasts after 15 days of incubation, while treated samples of a wild *S. cerevisiae* isolate were only slightly affected (Fig. 3.13). In the case of each GM yeast, the 0.15% treatment had a slightly greater effect on cell viability than the 0.3% treatment. However, this difference was not significant. Further testing involving longer periods of incubation will reveal whether the yeasts are able to acquire some resistance to the fungicide. The observed sensitivity of the GM yeasts to Ridomil could, however, explain the low amounts of yeasts isolated from the grapevines.

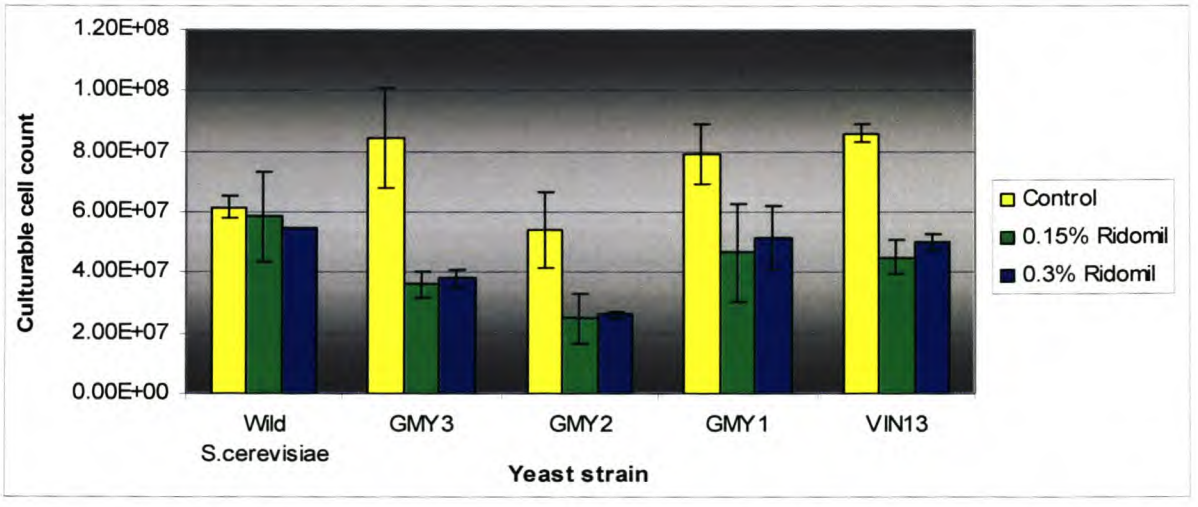

Fig. 3.13 The effect on culturable cell counts after treatment of the yeast isolates with a fungicide for 15 days.

3.3.3 DETECTION OF GM YEASTS IN THE SOIL AND ON THE BARK

Soil and bark were monitored during autumn and winter of 2000, 2001 and 2002 for the presence of any GM yeasts. Although sonication of the soil was experimented with, normal washing of the soil in 0.9% NaCl solution resulted in good yeast isolation. Many yeast-like colonies were isolated and replica plated onto Lysine and selective assay media for different GM yeasts. No GMY1 was detected in any soil sampled from blocks (GMY1) and (VIN13+GMY1) in 2000. It was difficult to assess all the yeast-like colonies, since, despite the use of a fungus inhibitor, filamentous fungal growth was still apparent. Five months after inoculation (January 2002), GM yeasts were isolated from soil sampled from blocks (GMY1+GMY2) and (GMY1+GMY2+GMY3) (Table 3.2). GMY2 was most often isolated from soil, while no GMY3 could be detected. Overall, the GM yeasts were seldom isolated, and when present, they were found in low numbers. This is consistent with the behaviour of VIN13 and GMY1 observed in the environmental studies that will be discussed in

Chapter 4. In the latter, VIN13 and GMY1 introduced to a typical vineyard soil were seldom isolated in statistically significant numbers over a period of five months.

Table 3.2 Culturable GM yeasts isolated from the soil in the inoculated blocks in 2002.

2002	Block	GM yeast detected	CFU/ml/g
07-Jan	(GMY1+GMY2)	GMY2	3.40E+01
30-Jan	(GMY1+GMY2+GMY3)	GMY1	3.71E+02
	(GMY1+GMY2+GMY3)	GMY2	3.03E+02

Non-*Saccharomyces* species e.g. *Lipomyces kononenkoae* are known to express α-amylase (Spencer-Martins and Van Uden, 1977; Horn *et al.*, 1988). Therefore, in terms of risk assessment, the introduction of a yeast with added α-amylase expression to a soil environment (such as the case of GMY1) will not present an unnatural condition, as many wild-type yeasts in the soil also express α-amylase.

In January 2001, small pieces of grapevine branches were monitored before the grapevines were pruned. No *S. cerevisiae* or GM yeast could be detected on any of the branches or bark sampled.

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CHAPTER 4

RESEARCH RESULTS

Behaviour of GM yeasts in fermentations

4. RESEARCH RESULTS

4.1 INTRODUCTION

Much effort has been directed towards the genetic improvement of yeasts that could be of considerable value for the brewing, winemaking and baking industries. In the past decade, recombinant DNA technology has been successfully applied to industrial yeast strains by increasing the possibilities of introducing new features and has resulted in the development of a new generation of specialised industrial yeast strains. Some of the significant advances are improved process performance, elimination of off-flavours, increased formation of desirable by-products, improvement of hygienic properties and the extension of substrate utilisation (Dequin, 2001).

Although *Saccharomyces cerevisiae* has been a favourite host for the expression of heterologous proteins of biotechnological importance, it is not recognised as a significant producer of extracellular depolymerising enzymes, such as pectinases, amylases and cellulases, which can make fermentable sugars available from the huge energy reserves present in biomass sources like pectin, starch and cellulose. For the bioconversion of polysaccharide-rich biomasses, commercial enzyme preparations and/or co-cultures are therefore being utilised. As an alternative to this approach, *S. cerevisiae* can be exploited to express heterologous polysaccharide-degrading enzymes to make a direct one-step bioconversion of polysaccharides possible (Van Rensburg *et al.*, 1994).

Enzymes have a significant role in the production of wine (Van Rensburg and Pretorius, 2000). The main polysaccharides responsible for turbidity, viscosity and filter stoppages in winemaking originate from the grapes, the fungi on the grapes and the microorganisms present in the winemaking process. These polysaccharides are pectins, glucans (a component of cellulose) and hemicellulose (mainly xylans) (Pretorius, 1997). In the winemaking process, exogenous industrial enzyme preparations are widely used to aid the endogenous pectinase, glucanase, xylanase and arabinofuranosidase activities of the grapes and yeast (Colagrande *et al.*, 1994). A wide variety of heterologous glucanases, pectinases, xylanases and arabinofuranosidases have already been expressed in *S. cerevisiae*, since the endogenous enzymes produced by *S. cerevisiae* are not sufficient to avoid the problems caused by polysaccharides in wine (Pretorius, 2000). In future, the use of heterologous wine yeast strains, such as GMY2 and GMY3 could aid the clarification of wine and minimises the levels of commercial enzyme preparations needed.

The spontaneous fermentation of grape juice can be seen as a heterogeneous microbiological process involving the sequential activities of various microbial species, mainly consisting of yeast flora that are "naturally" present in the

environment (Pretorius, 2000). Since *S. cerevisiae* is so scarcely found on grape surfaces, its growth in must becomes apparent only after four to five days of fermentation (Martini *et al.*, 1996). In must, the selective pressure during fermentation favours yeasts with the most efficient fermentative ability, which are strains of *S. cerevisiae* (Pretorius, 2000). In the early stages of fermentation, the genera *Kloeckera, Hanseniaspora* and *Candida* predominate. In the middle stages, when the ethanol levels rise to 3-4%, *Metschnikowia* and *Pichia* are predominant until strains of *Saccharomyces* take over in the latter stages (Pretorius *et al.*, 1999).

In this part of the thesis the fermentative ability and relative abundance of GMY1, GMY2 and GMY3 were monitored in a fermentation environment. Spontaneous fermentations were performed with grapes harvested from the glasshouse at certain intervals during the season. Small-scale fermentations were also performed in must of a different grape variety with inocula of mixed yeast resembling the inoculated yeasts in the glasshouse.

4.2 MATERIALS AND METHODS

4.2.1 SPONTANEOUS FERMENTATION OF INOCULATED GRAPE MUST

On three separate occasions during the monitoring seasons of 2000 and 2001, approximately 1 kg of grapes from each of the four representative blocks was harvested, crushed and spontaneously fermented. Fermentations were performed at room temperature in standard 750 ml wine bottles filled with approximately 600 ml must (resulting from a kilogram of grapes) and equipped with air locks. The weight loss resulting from escaping CO_2 was followed until the end of fermentation. Every second day, samples were drawn aseptically for the determination of a total yeast count. The bottles were carefully shaken by hand prior to each sampling. One millilitre of sample was serially diluted and spread inoculated in duplicate onto YM agar plates. The plates were incubated at 30°C for two to three days. Subsequently, colonies were replica plated onto Lysine medium and the different assay media to enumerate the number of GM yeasts detected in the fermentations. Due to the limited number of grapes on the young grapevines, each fermentation could be performed only once.

4.2.2 CONTROLLED FERMENTATION WITH GM YEASTS

Controlled fermentations were performed on a mini-scale with a known inoculum of yeasts mimicking the same combinations of yeasts inoculated onto the grapevines in the glasshouse. Precultures of the yeasts were grown overnight in YPD medium, then washed with water and resuspended in water to be used as inocula. A cell density of 1 to 3E+06 CFU/ml is usually recommended for a starter culture to dominate the native yeast species present in grape must (Fugelsang, 1997). The

formula used to determine the amount of yeast necessary for inoculation at this concentration was: $0.05/OD_{600}$ (optical density measured at 600 nm) x volume of must (ml) used to inoculate into. Yeasts were inoculated at a concentration of 3.00E+06 CFU/ml into 500 ml of Claret Blanc must. Fermentations performed with VIN13 and GMY1, as well as with the control, were done in duplicate. Fermentations with the combinations of (VIN13+GMY1), (GMY1+GMY2) and (GMY1+GMY2+GMY3) were performed in triplicate. All combinations of yeasts were inoculated at a ratio of 1:1 or 1:1:1. The fermentations were treated in the same way as previously described for the spontaneous fermentations (see section 4.2.1).

4.2.3 STATISTICAL ANALYSIS

The statistical analysis of all the fermentations that were performed included analysis of variance (ANOVA), post hoc analysis with the Bonferroni-Dunn calculation and was performed by using Statistica v6.0.1 software (StatSoft, Inc.). The significance levels for the *P* values are annotated in the figures.

4.3 RESULTS AND DISCUSSION

4.3.1 SPONTANEOUS FERMENTATION OF BERRIES HARVESTED IN GLASSHOUSE

In 2000 and 2001, the berries from the grapevines in the different blocks were harvested on a small scale, crushed and left at room temperature to spontaneously ferment. The berries were harvested at three different stages during each season, namely once in October and twice in November. All the fermentations showed the expected pattern of accumulated weight loss due to loss of CO_2 production (Fig. 4.1A and 4.1C). The controls only reached peaked fermentation after 12 days. The third control fermentation in 2000 was stuck, since very little weight loss occurred (results not shown). This fermentation was performed in late November, when the berries were very ripe with evidence of rotting. Stuck or sluggish fermentations present enormous practical problems in winemaking and occur when fermentation does not proceed to completion and an unacceptably high amount of residual sugars remains in the wine (Bisson, 1999). Stuck fermentations have often been observed in must derived from newly established vineyards, especially those in geographical areas that have never before been cultivated with grapes and therefore probably contain soils that do not yet harbour specialised, fermenting yeast cells (Martini *et al.*, 1996). For the work described here, 2000 was only the second year in which the grapevines carried grapes and this may explain the occurrence of a stuck fermentation with berries from the control block. The fact that it did not occur

with any of the fermentations from the inoculated blocks, confirms the presence of the inoculated yeast.

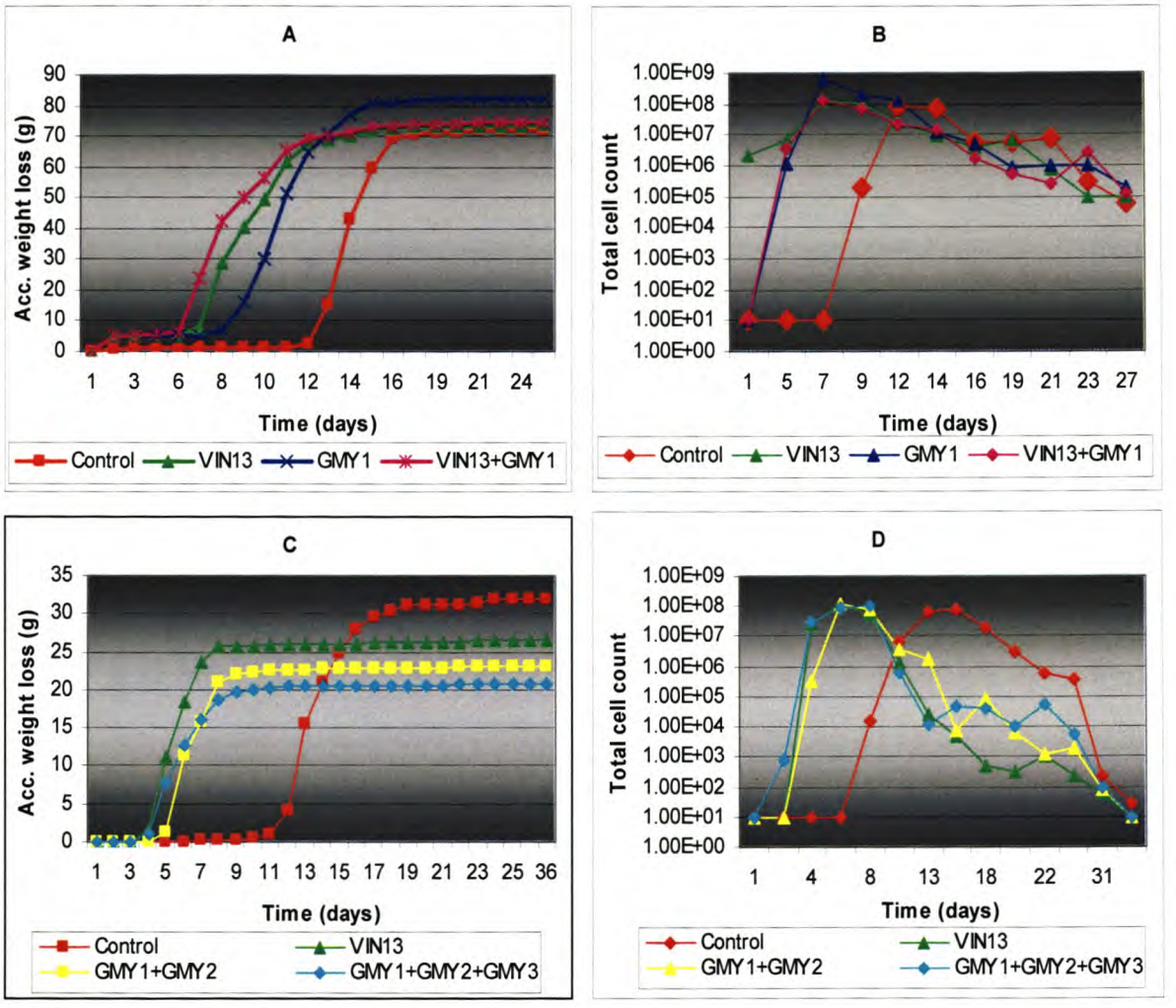

Fig. 4.1 Fermentations performed with the berries harvested from different treated blocks in the glasshouse. (A) The accumulated loss in weight of fermentations performed in 2000. (B) Total culturable cell count of fermentations performed in 2000. (C) The accumulated loss in weight of the fermentations performed in 2001. (D) Total culturable cell count of fermentations performed in 2001. The representative fermentation shown in each case is from berries harvested in early November.

The normal course of fermentation was observed in all four fermentations in both 2000 (Fig. 4.1B) and 2001 (Fig. 4.1D). Total cell counts during the spontaneous fermentations were as expected, except in the case of the controls, where fermentations commenced early and the rate was faster presumably due to the higher dosage of yeasts present on the berries. A determination of the inoculated yeasts in the total cell counts of each fermentation revealed that these yeasts were present on the grapes throughout the season. These results indicate that the inoculated GM yeasts survived on the berries. No significant differences were observed between the fermentations of berries that were sprayed with VIN13, GMY1 or the different combinations of yeasts: (VIN13+GMY1), (GMY1+GMY2) and (GMY1+GMY2+GMY3). The anomalous high count for VIN13 on day 1 (Fig. 4.1B)

can possibly be explained by the higher amount of inoculated VIN13 present on the specific berries that were randomly sampled. The natural fermenting ability of the unmodified strain, VIN13, was conserved in the recombinant strains, GMY1, GMY2 and GMY3.

At selected intervals, the relative abundance of the GM yeasts was determined in the fermentations of berries from block (VIN13+GMY1) in 2000 (Fig. 4.2) and (GMY1+GMY2) in 2001 (Fig. 4.3). Yeasts were expressed as a percentage detection in relation to each other. Statistical analysis of the spontaneous fermentation data from 2000 shows that in only one instance (berries harvested in early November), did GMY1 outperform VIN13 in terms of cell numbers (Fig. 4.2D; $P = 0.00002$). There was no statistical difference in the numbers of these two strains in fermentations with berries that were picked in early October ($P = 0.35$) and late November ($P = 0.093$) (Fig. 4.2B and 4.2F, respectively). A similar trend was observed during the fermentations performed in 2001; only fermentations with berries picked in early November showed a significant difference between GMY1 and GMY2. As for 2000, the difference occurred at day eight (Fig. 4.3D).

With the exception of these sampling dates, the data suggest that the genetic modifications applied to these strains did not confer a competitive advantage. A plausible explanation for the observed differences in relative abundance during the early stages of fermentations is that the cell loads on the berries were different, and that these differences were obliterated with time in the absence of a competitive advantage by any of the strains. The assay for detecting GMY3 was found to be not consistent; therefore the results for GMY3 are not included. To address this, and to negate the possible effect of inoculum size, small-scale fermentations with inocula of mixed yeast were performed.

4.3.2 CONTROLLED FERMENTATION

To determine the relative fitness of the different GM yeasts when subjected to direct competition, fermentations were conducted with mixed inocula. An array of fermentations with combinations of yeasts that were representative of the combination of yeasts inoculated onto the grapevines in the glasshouse was performed in triplicate. As expected, the control started fermenting later and less vigorously in comparison to the rest of the fermentations (Fig. 4.4A). Only after day two did the fermentation rate of the control increase, while the other fermentations started fermenting vigorously from day one. The total cell count for the control was also never as high as for any inoculated fermentation (Fig. 4.4B). All the fermentations followed the same general pattern.

At the same intervals at which total cell counts were determined, all *S. cerevisiae* colonies resulting from fermentations performed with the combinations of yeasts were assessed according to the appropriate GM yeast assay media (Chapter 3,

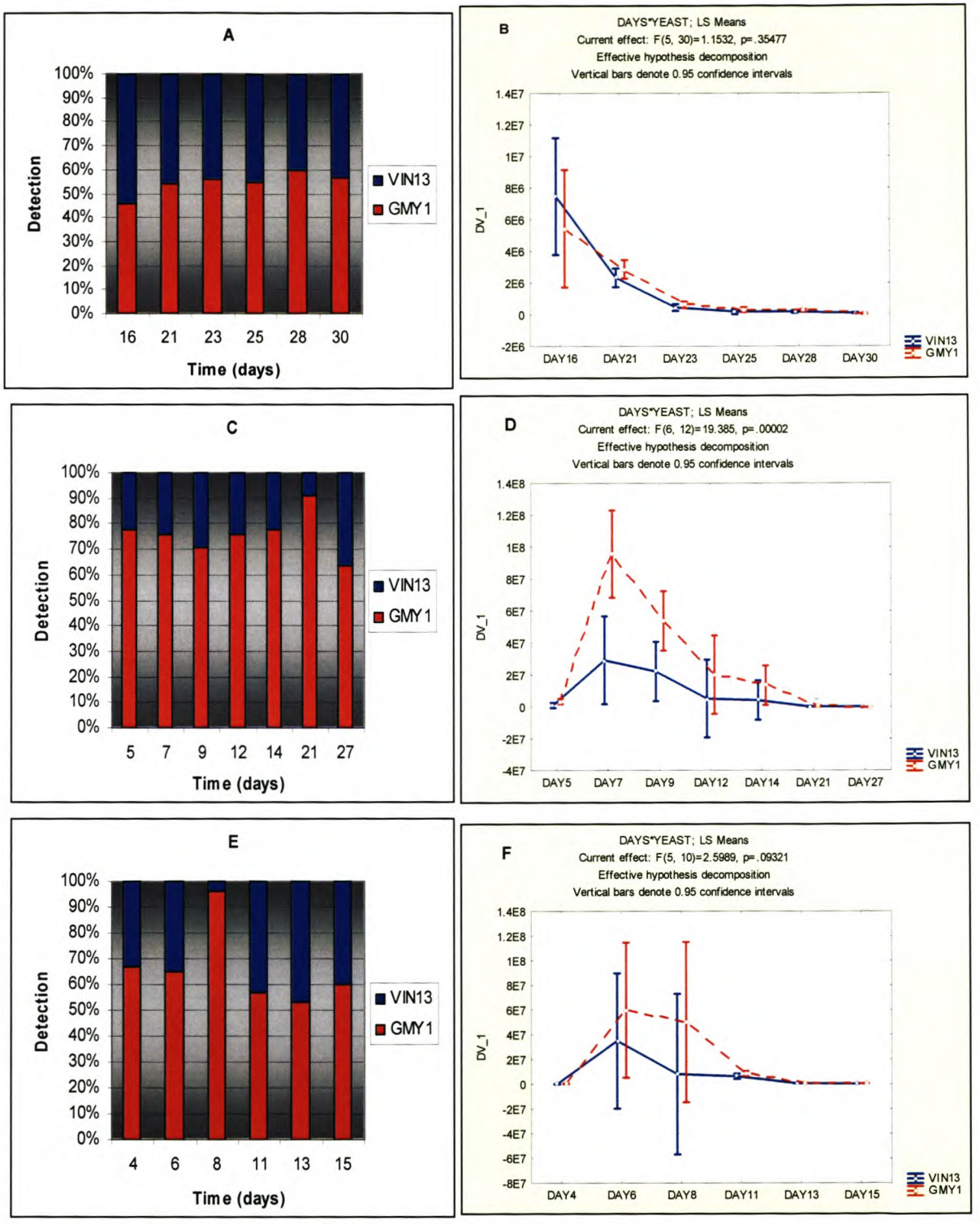

Fig. 4.2 The detection of VIN13 and GMY1 in spontaneous fermentations performed with berries from block (VIN13+GMY1). Three fermentations were performed at different times during the season of 2000: early October (A), early (C) and late November (E). The statistical analysis of the behaviour of the yeasts in the aforementioned fermentations is indicated in (B), (D) and (F), respectively.

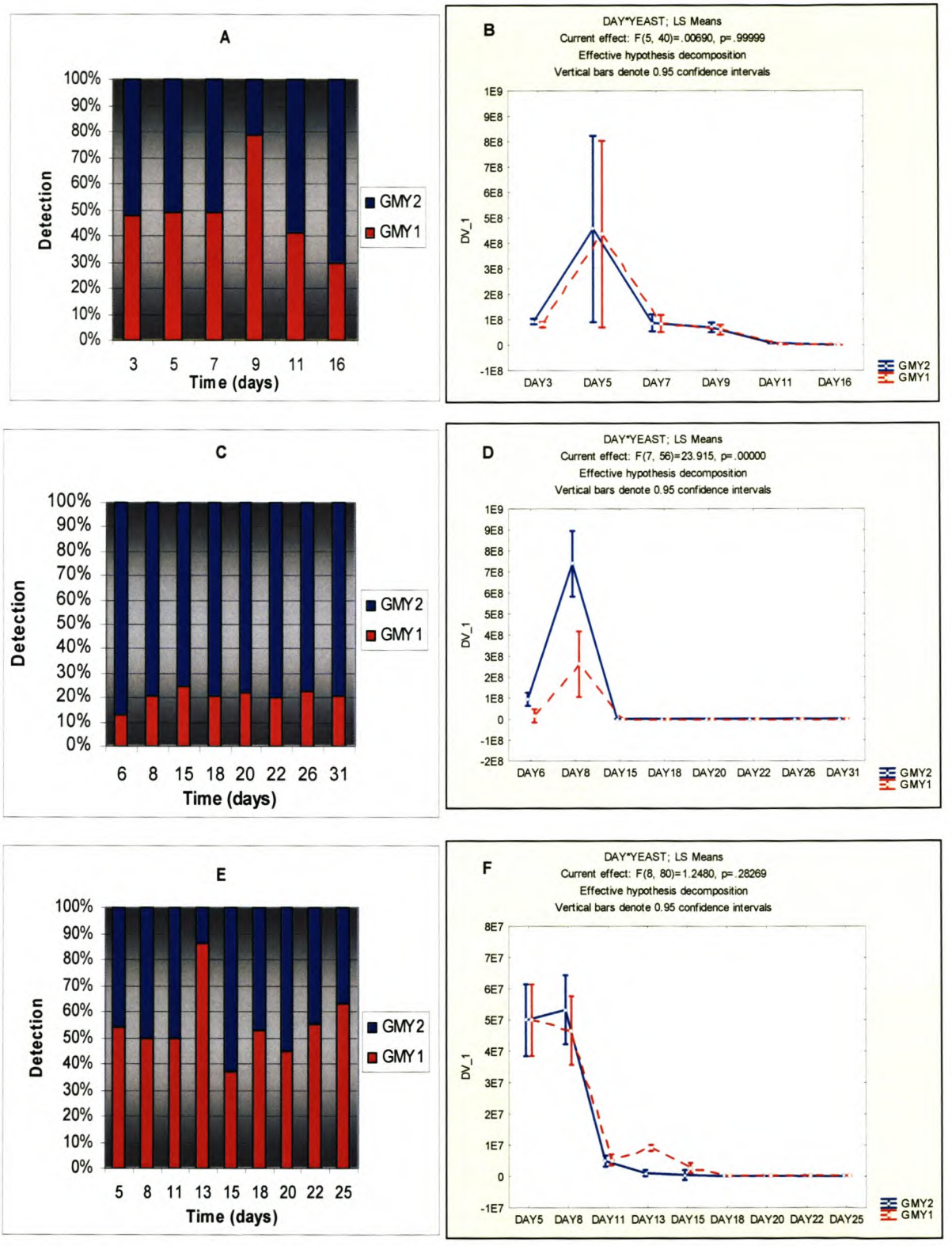

Fig. 4.3 The detection of GMY1 and GMY2 in spontaneous fermentations performed with berries from block (GMY1+GMY2) in 2001. Three fermentations (A, C and E) were performed in the same manner as for 2000 (Fig. 4.2). The statistical analysis of the behaviour of the yeasts in the different fermentations is also indicated in (B), (D) and (F), respectively.

section 3.2.6). The amounts of detected GM yeasts were then expressed as a percentage of each other. Statistical analysis of the fermentations was also performed. Significant differences in the fermenting behaviour of VIN13 and GMY1 could be observed in fermentation (VIN13+GMY1) (Fig. 4.5A and 4.5B). Although VIN13 and GMY1 were inoculated at a ratio of 1:1, they were not equally detected throughout the fermentation. Much less GMY1 was detected from the start and further decreased from day six onwards. At most, GMY1 represented ~35% (day eight) of the total *S. cerevisiae* colonies assayed. There was a significant difference (P = 0.00004) between the behaviour of VIN13 and GMY1 for the duration of the fermentation (Fig. 4.5B). Due to the fact that fermentation (VIN13+GMY1) was performed by VIN13 together with GMY1, it might be speculated that GMY1 was present in greater numbers than detected because of a loss of α-amylase activity. It is possible that GMY1 may have lost expression of its α-amylase because it conveys no competitive advantage in a fermentation environment and becomes a metabolic burden for the yeast. The loss of α-amylase activity was however not confirmed by PCR or Southern blotting. This behaviour by GMY1 was observed in all the combinations of yeast fermentations (Fig. 4.5A, 4.5C and 4.5E).

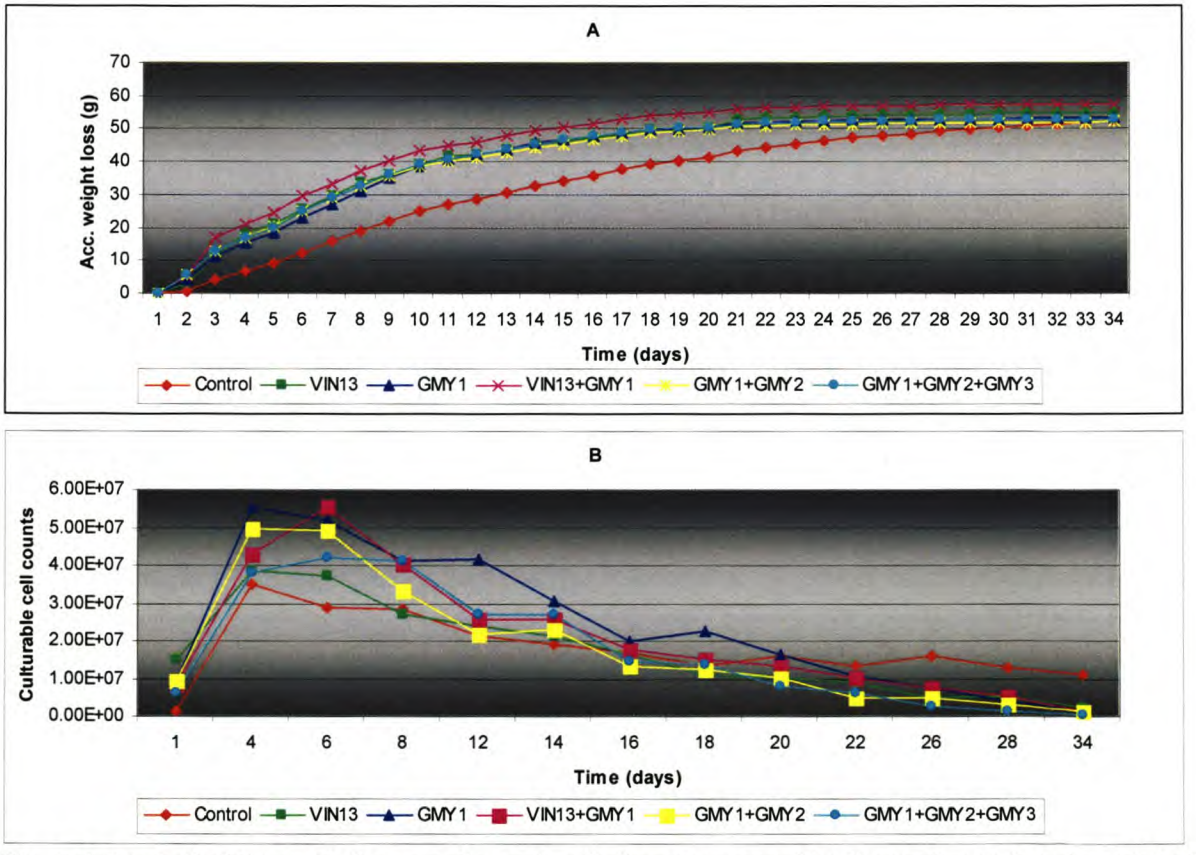

Fig. 4.4 Controlled fermentations performed with the same combinations of yeast as inoculated on the grapevines. (A) The accumulated loss in weight measured at intervals. (B) The average total culturable cell counts for the yeasts.

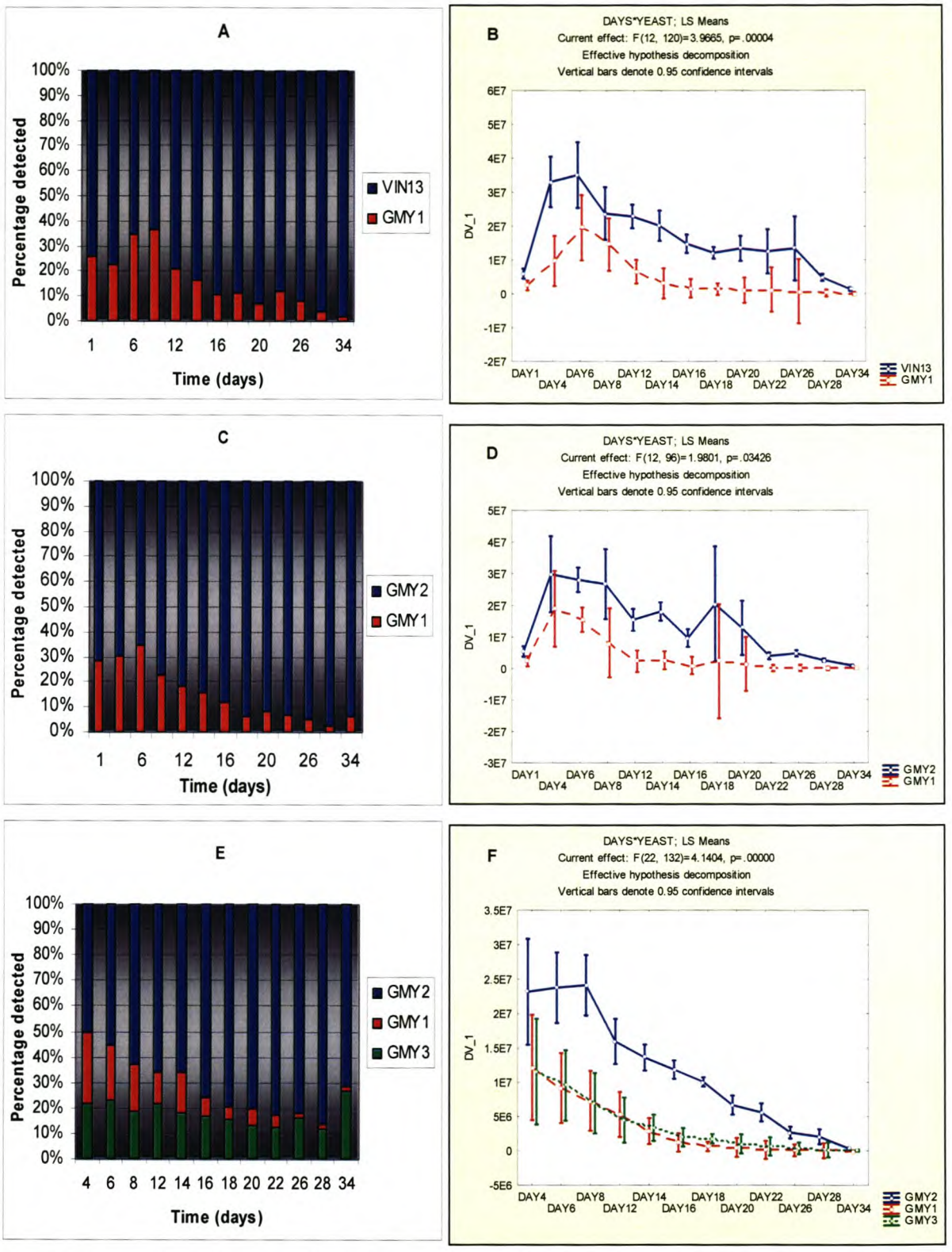

Fig. 4.5 Detection of yeasts from the controlled fermentations performed by the different combinations of yeasts inoculated. (A) The percentage detection of VIN13 in relation to GMY1 where both yeasts were co-inoculated. (C) The percentage detection of GMY1 in relation to GMY2 where both yeasts were co-inoculated. (E) The percentage detection of GMY1, GMY2 and GMY3 in relation to each other where all three yeasts were co-inoculated. The statistical analysis of the fermentations is shown in (B), (D) and (F), respectively.

Fermentation (GMY1+GMY2) behaved in a similar way to fermentation (VIN13+GMY1). Of the total *S. cerevisiae* colonies assayed, GMY1 was detected at the most ~35% (day six) when it was co-inoculated with GMY2 (Fig. 4.5C). The amount of detected GMY1 gradually decreased from day six onwards. In comparison to fermentation (VIN13+GMY1), it seems that GMY2 behaved in a similar way to VIN13 towards GMY1. Statistically, GMY1 and GMY2 performed significantly ($P = 0.03$) different for the duration of the fermentation (Fig. 4.5D).

In fermentation (GMY1+GMY2+GMY3), GMY2 behaved differently from both GMY1 and GMY3 (Fig. 4.5E). GMY2 gradually became dominant from day four onwards. The numbers of GMY1 gradually decreased from day four onwards, and very low numbers of GMY1 were detected after day 22. The presence of GMY3 also decreased, with slight fluctuations at the beginning and end of the fermentation. GMY3 was detected at less than 30% of any daily total of *S. cerevisiae*. Statistically, it was obvious from Fig. 4.5F that GMY2 behaved differently to GMY1 and GMY3 over the duration of the fermentation, whereas the latter two yeasts displayed similar behaviour. It is obvious from these results that GMY2 had a competitive advantage in fermentation where all three GM yeasts were co-inoculated. This could be explained by GMY2 expressing both an endo-β-xylanase and an endo-β 1,4-glucanase in the must, that presented an optimal environment for this yeast. It is interesting that GMY3 was detected in such low numbers, especially in light of the fact that this GM yeast expresses a secreted pectate lyase and a polygalacturonase that should, in theory, have given the yeast an advantage in a must environment. It is possible that all the other yeasts present in the fermentation benefit from the secreted enzymes, while GMY3 pays the cost of their synthesis. The expression of the combination of these two genes might put too much of a metabolic burden on GMY3 in this specific must environment. Another possibility is the chance that differences in ethanol tolerance of these yeasts could have developed as a result of the genetic modifications.

The comparison of the spontaneous and controlled fermentations revealed contradicting results. In spontaneous fermentations performed with berries from block (VIN13+GMY1) and (GMY1+GMY2), only one out of three fermentations indicated a significant difference in the overall behaviour of the inoculated yeasts (see Fig. 4.2 and 4.3). Interestingly, the fermentation indicated as different in both cases was performed with berries harvested during the same time in the season (early November). Fermentations performed with inocula containing equal numbers of each yeast (VIN13+GMY1) or (GMY1+GMY2) revealed a significant difference in the overall behaviour for both combinations (see Fig. 4.5A to 4.5D). Even when GMY2 was co-inoculated with the other GM yeasts, it proliferated in a fermentation environment (Fig. 4.5D and 4.5E). It is most likely that the dosage response of the inoculated glasshouse berries, as well as the type of must, could have had a significant influence on the amount of GM yeast detected.

The utilisation of these GM yeasts in the industry could have some implications with regard to the expression of the specific transgenes in different types of must. It might be possible that certain GM yeasts will not perform equally well in all types of must and will only be suitable for the production of certain kinds of wine varieties.

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CHAPTER 5

RESEARCH RESULTS

Monitoring of the genetically modified yeast, *Saccharomyces cerevisiae* VIN13 expressing an α–amylase, in model systems

5. RESEARCH RESULTS

5.1 INTRODUCTION

The budding yeast, *Saccharomyces cerevisiae,* has enjoyed a long and distinguished history in the fermentation industry. Owing to its fermentation efficiency in producing alcohol and gas, *S. cerevisiae* is without doubt the most important commercial microorganism with GRAS (Generally Regarded As Safe) status (Pretorius *et al.*, 2003). By brewing beer, making sparkling wine and leavening dough, mankind's oldest domesticated organism made possible the world's first biotechnological processes. With the emergence of genetic engineering in the 1970s, *S. cerevisiae*, as one of the most popular scientific models and commercially important microorganisms, has played a leading role in (i) shifting the frontiers of DNA science; (ii) increasing our understanding of fundamental cellular and molecular processes; and (iii) the application of modern biotechnological tools in industry. In fact, the industrial importance of *S. cerevisiae* has extended beyond traditional fermentation. Today, the products of yeast biotechnologies impinge on many commercially important sectors, including food, beverages, biofuels, chemicals, industrial enzymes, pharmaceuticals, agriculture and the environment.

The first approved human vaccine (against hepatitis B) and food product (calf chymosin for cheese making) resulting from recombinant DNA technology were produced with transgenic *S. cerevisiae* strains (Pretorius *et al.*, 2003). *S. cerevisiae* was also the first genetically modified *organism* (GMO), as distinguished from a genetically modified (GM) *product*, to be cleared in the 1990s for food use, as a baking and brewing strain (Walker, 1998). The GM baking strain containing constitutively expressed maltose permease and maltase genes, produces CO_2 faster than conventional baker's yeasts, thereby ensuring that dough rises more rapidly (Smith, 1998). The novel engineered feature of the "pioneer" GM brewer's yeast is a glucoamylase-encoding gene that allows partial hydrolysis of maltodextrins, yielding a lower-carbohydrate beer (Smith, 1998). Recently, a wine yeast has been developed that is capable of converting malic acid in grape must either to ethanol (malo-ethanolic fermentation) or to lactic acid (malolactic fermentation) during alcoholic fermentation of the winemaking process (Pretorius, 2003). These recombinant yeasts circumvent problems with sluggish or stuck malolactic fermentations conducted by lactic acid bacteria. The "malolactic yeast" was the first wine yeast to be commercialised by a yeast manufacturing company and it has been trialled in 2002/2003 in Moldavia. This represents the first large-scale (20000-litre) winemaking trial with a GM wine yeast (Pretorius and Høj, 2005).

In addition to the aforementioned "*first*" examples of the genetic improvement of industrially important strains of *S. cerevisiae*, further efforts have been made in recent years to develop superior yeast strains for the fermentation industry. The main programmes for yeast strain development have been, and still are, the

improvement of fermentation performance, processing efficiency, sensory quality and wholesomeness of the end-product (Pretorius and Bauer, 2002). Numerous useful GM yeast strains already exist in laboratories, while many others are being constructed. Although some of these GM yeasts comply with the strict legislation of most countries and have been cleared by regulatory authorities for commercial use, GM yeasts have not, as yet, been used for the commercial production of GM bread, beer or wine. The potential benefits of GM foods have so far largely been outweighed by the public's perception of the potential risks associated with these foods (Vivier and Pretorius, 2002). Nevertheless, the use of GM yeasts on the market appears imminent and there is an urgent need to assess and address the perceived health and environmental risks associated with GM foods (Bauer *et al.*, 2004).

Although the concept of "substantial equivalence" is used in the determination of safety, it is not a safety assessment in itself, but is used to structure the assessment of the safety of a GMO relative to the unmodified form (Thomson, 2002). Generally, an appropriate risk assessment procedure is an essential prerequisite when the use and release of any GMO into the environment is considered. One approach to risk assessment is to compare the relative fitness of the GM strain with that of the wild type and any differences then become the focus of the safety assessment.

Microbial communities have been shown to associate with surfaces in aqueous and terrestrial environments. This mode of growth is generally referred to as a biofilm and defined as microbial populations adherent to each other and surfaces or interfaces (Costerton *et al.,* 1995). Biofilm formation is a universal microbial strategy for survival and optimum acquisition of available nutrients. Microbial biofilm populations produce detached, planktonic cells with less chances of survival, but also with the capacity to colonize new surfaces or form large planktonic populations in those rare nutrient-rich environments where microbial antagonists are few (Costerton *et al.*, 1987). Biofilms have been implicated in many microbial processes, e.g. tooth decay and biofilm reactors in wastewater treatment. Therefore, biofilms are often exploited as model systems to study bacterial behaviour.

To date, research on biofilm formation has focused primarily on bacteria, while yeast-biofilm research is mostly related to *Candida* spp. Many of the fungi that are involved in biofilm formation are not responsive to genetic approaches and this is one of the main reasons why there is still relatively little known about fungal biofilms (Baillie and Douglas, 1999; O'Toole *et al.*, 2000). In search of a model system for fungal biofilms, Reynolds and Fink (2001) have shown that *S. cerevisiae* can initiate biofilm formation on a number of plastic surfaces. It was suggested that active metabolism is a requirement for adherence, since growth in a lowered glucose concentration enhanced the adherence of yeast to plastic surfaces, while the total absence of glucose led to a reduced adherence. The expression of *FLO11* in *Saccharomyces* might play a similar role in adherence to the glycopeptidolipids (GPLs) expressed on the cell surface of the non-flagellated bacterium,

Mycobacterium smegmatis. These GPLs are thought to be required for biofilm formation, as well as "sliding motility" behaviour, because they increase surface hydrophobicity (Recht *et al.*, 2000). It is known that filamentous growth in *Saccharomyces* is dependent on the *FLO11* gene. This form of growth is induced by conditions of nitrogen starvation where a switch from the yeast-like form to invasive chains of elongated cells or pseudohyphae occurs (Liu *et al.*, 1993; Roberts and Fink, 1994; Rupp *et al.*, 1999). The role of *FLO11* in the adherence of yeast cells to plastic, and the phenomenon of invasive growth can possibly be explained by Flo11p promoting both cell-cell adhesion and cell-surface adhesion. As a cell surface protein, Flo11p may have distinctive properties that enable it to initiate biofilm formation (Reynolds and Fink, 2001).

The soil environment forms an important part of the biosphere and the transport and attenuation of a GM yeast in this matrix will to a large extent affect their ultimate fate in the environment. In soil, microorganisms either occur as suspended cells in pore water or as biofilms on soil surfaces. Growth conditions in the soil environment differ substantially from those that are typically applied in the laboratory during the cultivation of microorganisms. While the latter often involves pure cultures and rich growth media, the models used in this study were selected to provide a closer representation of the natural ecosystem.

Despite the widespread use of *S. cerevisiae* in industry and in science, relatively few studies have addressed biofilm formation by this organism, or its environmental fate. In spite of efforts to find *S. cerevisiae* in nature, it is seldom found on healthy, undamaged grapes and also rarely isolated from vineyard soils (Martini, 1993). There is however a school of thought that the primary source of *S. cerevisiae* is indeed the vineyard, with its occurrence differing entirely with each plant and grape cluster (Török *et al.*, 1996). Mortimer and Polsinelli (1999) proposed that damaged grape berries are rich depositories of microorganisms which include *S. cerevisiae*, and that these microorganisms are inoculated into the interior of damaged berries by insects. Others are of the opinion that a natural origin for *S. cerevisiae* should be excluded and that this species originated from the hybridisation of other *Saccharomyces* species that were then selected in man-made environments. A direct association of *S. cerevisiae* with man-made and artificial environments such as wineries are well known (Martini, 1993). *S. cerevisiae* has in fact seldom been isolated from areas that are not closely associated with humans (Naumov and Naumova, 1991; Naumov, 1996; Naumov, 1998).

In this part of the thesis the behaviour of GMY1, designated LKA1, the wild-type VIN13, as well as environmental bacterial and yeast strains, was studied under different scenarios. To evaluate the transport of VIN13 and LKA1 through a soil matrix, sand columns were set up and breakthrough curves measured. Continuous-flow cells were subsequently used as model systems in combination with epifluorescence and scanning electron microscopy (SEM) to determine if the strains formed biofilms in the presence of sand and glass surfaces. It is known that

microorganisms adhere to surfaces and grow as sessile biofilm communities in response to environmental stress conditions e.g. oligotrophic conditions. The flow cell experimentation was thus an attempt to visualise and verify the results obtained in the soil columns.

5.2 MATERIALS AND METHODS

5.2.1 STRAINS USED

S. cerevisiae VIN13, and the genetically modified VIN13, LKA1, were used as examples of industrially relevant strains. *Cryptococcus laurentii* was used as an example of widely occurring environmental yeast. The yeasts *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii* and *Kloeckera apiculata*, and the bacterial isolates CT01, CT04, CT05 and CT08 were included to establish a mixed biofilm community. Sequencing of the 16S rDNA of bacterial isolates CT01, CT04 and CT08 (in both directions to completion) revealed the respective nucleotide identities as follows: *Microbacterium nematophilum, Dyadobacter fermentens* and *Pseudomonas* sp. AEBL3. CT05 is still unidentified.

The *LKA1* gene was expressed in *S. cerevisiae* VIN13 under the control of the phosphoglycerate kinase (*PGK1*) promoter and terminator, and integrated into the yeast genome via the sulfometuron methyl resistance marker *(SMR1)*. *LKA1* was originally cloned from the yeast, *L. kononenkoae* (Steyn *et al.,* 1995) and encodes the raw starch degrading α-amylase that liberates reducing groups from glucose polymers containing both α-1,4 and α-1,6 bonds. LKA1 colonies were visualised as zones of raw starch degradation on Phadabas agar plates containing 6.7 g YNB with amino acids, 1 g glucose, 20 Phadabas tablets (Pharmacia Diagnostics) and 20 g agar per litre of distilled water.

5.2.2 YEAST BEHAVIOUR IN A POROUS MATRIX

5.2.2.1 Saturated sand columns under conditions of continuous flow

Polyethylene columns (diameter of 23 mm, length of 140 mm) were filled with sieved (1180 μm) autoclaved sand. The columns were pre-sterilised with 3.5% (v/v) sodium hypochlorite for 0.5 hours (h) and then rinsed for 4 h with sterile Ringers solution (Merck). An effort was made to minimise air bubble and channel formation while filling the columns. The total pore volume (PV) was determined for each experiment. Filters were cut from hybridisation mesh and placed at each end of the columns to prevent blockage at the columns' ends. Streptomycin sulphate (0.5 g/L) was added to the soil columns as needed to prevent bacterial contamination.

The inlet of each column was connected with silicon tubing (1.6 mm ID) to a Watson-Marlow 502S peristaltic pump and a reservoir containing sterile Ringers solution. The Ringers solution was pumped through the column at 2.3 ml/h. One PV of the culture suspended in Ringers solution was subsequently pumped through the

columns at the same flow, where after flow was resumed with Ringers solution. A BIO-RAD, model 2110 fraction collector was used to collect effluent samples every hour.

5.2.2.2 Preparation of cultures

Isolates were cultivated overnight at 30°C; VIN13 and LKA1 in 50 ml full-strength YM medium (containing 0.3% yeast extract, 1% glucose, 0.3% maltose, 0.5% peptone from casein) and *Cryptococcus* and *D. fermentens* in 50 ml 10% YM medium. Cultures were centrifuged for 5 min at 5000 rpm and washed three times with Ringers solution. Cells were then re-suspended in 150 ml Ringers solution and the cell suspensions introduced into the columns as described above. In addition to pure culture suspensions, co-cultures of *Cryptococcus* and LKA1 were simultaneously introduced into columns, in which case equal volumes of cultures were used. The initial cell concentration (C_0) was determined in each case by serial dilution and enumeration of viable cell numbers on YM agar plates.

5.2.2.3 Sampling methods

The total volume of selected samples (2.7 ml each) obtained from the fraction collector was centrifuged for 5 min at 5000 rpm, re-suspended in 200 µl Ringers solution and plated in duplicate onto YM agar plates containing streptomycin sulphate (0.5 g/L). Tryptone Soy agar (TSA) plates (Biolab) without streptomycin sulphate were used in the case of experiments with *D. fermentens*.

At the end of each experiment, the sand columns were dissected in 1 cm sections and the sand from each section aseptically removed to determine the penetration distance of the test organism. Each sand sample (~10 g) was added to 90 ml of Ringers solution in an Erlenmeyer flask, shaken by hand for 2 min and by Vortex (Vortex Genie 2, Scientific Industries) for 1 min at maximum speed. Samples were then serially diluted and 100 µl plated in duplicate onto YM agar plates containing streptomycin sulphate (0.5g/L) for yeast, and on TSA plates without streptomycin sulphate for *D. fermentens*. The plates were incubated for two to three days at 30°C.

5.2.3 SOIL MICROCOSMS AT 20% MOISTURE CONTENT

PVC containers (18 cm diameter) were filled with ~1.2 kg of typical vineyard soil (consisting of 1/3 peat moss, 1/3 bark, 1/3 crushed dust; pH KCL: 6.2; density: 550.8 kg/m^3) and inoculated with VIN13 and LKA1, respectively. Yeasts were cultivated overnight at 30°C in 100 ml YPD medium (containing 1% yeast extract, 2% glucose, 2% peptone from casein) and centrifuged at 5000 rpm for 5 min. Cells were washed twice with sterile distilled water and re-suspended in a volume of water to result in final soil moisture content of 20%. VIN13 and LKA1 were inoculated at a concentration of 4.20E+07 and 2.98E+07 CFU/ml, respectively. The soil was watered on a weekly basis to moisture content of 20% and sampled at several intervals. Sampling involved collection of 10 g composite soil aliquots from the upper

5 cm of soil in each container and added to Erlenmeyer flasks containing 90 ml Ringers solution. Serial dilutions were then prepared as described above and plated in duplicate onto YM agar plates supplemented with 12% ethanol and incubated at 30°C for two to three days. Yeast-like colonies were subsequently replica plated onto Lysine (Biolab) and Phadabas agar plates. After 14 weeks, the sampled soil was added to Erlenmeyer flasks containing 90 ml of either Colombard grape must or 1% YM medium for enrichment purposes. These Erlenmeyer flasks were incubated for one day at room temperature without shaking before serial dilutions were made and the yeast numbers determined on YM agar plates. The purpose of this experiment was to evaluate the long-term survival of LKA1 in a typical vineyard soil.

5.2.4 CONTINUOUS FLOW CELLS FILLED WITH SAND

To further investigate the behaviour of yeast in the sand columns, flow cells filled with sand were used to visualise yeast-sand interactions. The channels of Perspex flow cells (Wolfaardt *et al.*, 1994) were filled with autoclaved sand and covered with a glass cover slip (no. 1 thickness, 75 x 50 mm) (Fig. 5.1). Each flow cell channel was connected with autoclaved silicone tubing (1.6 mm ID) to a reservoir containing either Ringers solution or 1% YM medium. Each flow cell consisted of six flow channels with dimensions 310 mm length x 40 mm wide x 2.2 mm deep. These sand-filled flow chambers were sterilised for 1 h with 3.5% (v/v) sodium hypochlorite and rinsed overnight with sterile growth medium. Aliquots of 300 µl of overnight VIN13, LKA1 and *Cryptococcus* cultures grown in full strength YM medium (1% in the case of *Cryptococcus*) were used to inoculate duplicate flow chambers. The overnight cultures were washed once with sterile distilled water (5000 rpm for 5 min) before re-suspension in either Ringers solution or 1% YM medium. After 4 h, flow was resumed through the channels at a flow rate of 2.3 ml/h, using a Watson-Marlow 205S multi-channel peristaltic pump. One ml of effluent from each channel was collected at daily intervals for seven days, serially diluted and plated in duplicate onto YM agar plates to determine suspended cell numbers. Biofilms were allowed to develop for periods of three, five or seven days before the channels were stained with Calcofluor™ White M2R (Molecular Probes, Eugene, Oregon, USA) at a concentration of 25 µM. Images were randomly captured with epifluorescence microscopy at 600x magnification. Channels were also stained with the FUN-1™ yeast viability probe (Molecular Probes, Eugene, Oregon, USA) at a concentration of 40 µM after each experiment of biofilm development.

A Nikon Eclipse E400 epifluorescence microscope equipped with excitation/barrier filter sets of 465-495/515-555 nm (Texas Red) and 540-580/600-660 nm (FITC), as well as a multipass filter set for viewing DAPI, was used for *in situ* visualisation of biofilm formation on the sand. Images were captured with a COHU high performance CCD Camera (Model nr. 4912-5010/0000) and a Nikon (Coolpix 9909) digital camera. A Scanning Electron Microscope (LEO 1430 VP) was used to obtain high-magnification images of biofilm formation on sand

granules that were collected from the flow channels after four and 17 days, respectively. Samples were gold-coated with an Edwards S150A Sputter Coater.

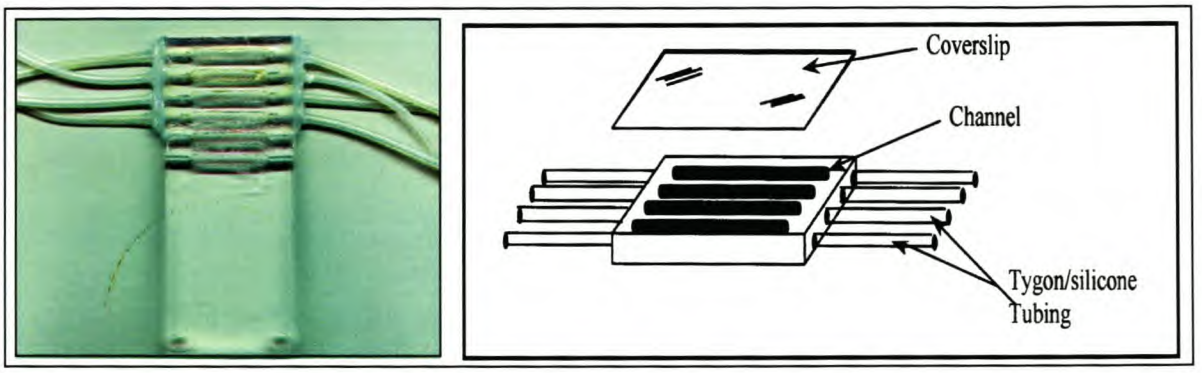


Fig. 5.1 A Perspex flowcell with flow chambers. A glass cover slip serves as the attachment surface for biofilm formation and is sealed onto the flowcell with silicone adhesive (Wolfaardt *et al.*, 1994). In the case where biofilm formation on sand particles was investigated, the flow chambers were filled with sand.

5.2.5 YEAST BEHAVIOUR IN AN AQUEOUS ENVIRONMENT

5.2.5.1 Biofilm formation by yeast isolates

Flow cells were also used to observe the behaviour of the test strains in a bulk aqueous environment. In this case, sterile flow cells were irrigated with 1% YM medium or 10% artificial winery effluent (AWE). The composition of AWE was 1.7 g YNB (Difco) without amino acids and ammonium sulphate, 5 g $(NH_4)_2SO_4$, 1.8 g glucose, 1.8 g fructose, 1 mg butanol, 1 mg citric acid, 2 mg malic acid, 2 mg tartaric acid, 2 mg lactic acid, 1.24 mg propanol, 3.8 mg i-amylalcohol, 0.25 g acetic acid, 4 mg ethyl-acetate, 8 mg propionic acid, 1 mg valeric acid, 0.5 mg hexanoic acid, 0.7 mg octanoic acid and 10 mg ethanol per litre, with the pH adjusted to 4 using NaOH. Individual flow chambers were inoculated in duplicate with 300 µl of overnight cultures of VIN13 and LKA1 grown in full strength YM medium and *Cryptococcus* grown in 1% YM medium. After washing once with distilled water, the yeast cultures were suspended in 1% YM medium to serve as inocula. When flow cells were irrigated with 10% AWE, the flow chambers were inoculated in duplicate with 400 µl of overnight cultures of VIN13 and LKA1 that were suspended in 10% AWE after washing. Flow of the irrigation medium was resumed at a flow rate of 2.3 ml/h after 4 h and kept at room temperature (22°C). Biofilms were stained with Calcofluor™ White M2R and FUN-1™ (Molecular Probes, Eugene, Oregon, USA) to determine cell viability using epifluorescence microscopy at the end of experiments. Standard procedures were followed to enumerate cell numbers in the effluent.

In addition to microscope analysis, a photometric approach was applied to quantify and compare biofilm formation rates between VIN13, LKA1 and *Cryptococcus*. Parallel-plate flow cells with an internal volume of ~12 ml were used to measure the accumulation of biomass on glass surfaces. In essence, this

approach measures light that passes through the flow cell. Biofilm accumulation is thus recorded in real time as a consequence of the lowered intensity of forward scattered light. Data was recorded at 5 min intervals for nine days and stored with a designated program executed in Lab View Student Edition, Version 3.1 (National Instruments). Preparation of flow cells and inoculation were performed as described for conventional flow cell experiments.

5.2.5.2 Behaviour of industrial strains in a biofilm community setting

Flow cells were irrigated with 10% AWE and replicate flow chambers inoculated with 400 µl of a mixed microbial community. The community consisted of 1 ml of an overnight culture of each of the yeasts *S. pombe*, *S. ludwigii and K. apiculata,* and the bacteria, *M. nematophilum,* isolate CT05 and *Pseudomonas* sp. AEBL3. The yeasts were cultivated in YM medium and the bacteria in 10% Tryptone Soy Broth (TSB) (Biolab) where after they were washed once in sterile distilled water and resuspended in 10% AWE. Flow was continued at a flow rate of 4.1 ml/h for five days before flow was arrested and 400 µl of an overnight culture of LKA1 inoculated in duplicate. Effluent was collected on days three, five (before the addition of LKA1) and ten, serially diluted, plated onto YM and Wallerstein Laboratory Nutrient agar plates (WL) (Difco) and incubated for four to five days at 30°C. Colony colour and morphology on WL nutrient agar plates were used to distinguish between the different yeast strains. On day ten, the diluted effluent was also plated on Phadabas agar plates to distinguish LKA1.

5.3 RESULTS AND DISCUSSION

5.3.1 BEHAVIOUR IN SAND COLUMNS

5.3.1.1 Behaviour of *S. cerevisiae* VIN13 and the GM strain *S. cerevisiae*, LKA1

No breakthrough of VIN13 or LKA1 cells occurred through the sand columns, and even after passage of up to 36 PV (~290 h), yeast cells could not be detected in the effluent collected during replicate experiments. The columns were dissected in 1 cm sections at the end of each experiment to assess the fate of the cells in the saturated sand. The majority of cells were found in the first 6 to 7 cm of the columns and did not spread further than 11 cm from the column inlet (Fig. 5.2).

5.3.1.2 Comparison of modified and unmodified *S. cerevisiae* to the soil yeast, *C. laurentii* and a bacterium, *D. fermentens*

A typical soil yeast, *C. laurentii* and a bacterium, *D. fermentens*, were used to compare the behaviour of VIN13 and LKA1 in the sand matrix. Duplicate column experiments with *Cryptococcus* showed a rapid breakthrough by this yeast; in both cases a breakthrough of cells was observed after 1 PV (~8 h) (Fig. 5.3A). This figure also shows that when the modified *S. cerevisiae,* LKA1, was co-inoculated with

Cryptococcus, the latter had a similar breakthrough (migration through the sand profile) than when introduced in pure culture, indicating that the presence of the *Saccharomyces* did not have an influence on the movement of *Cryptococcus* through

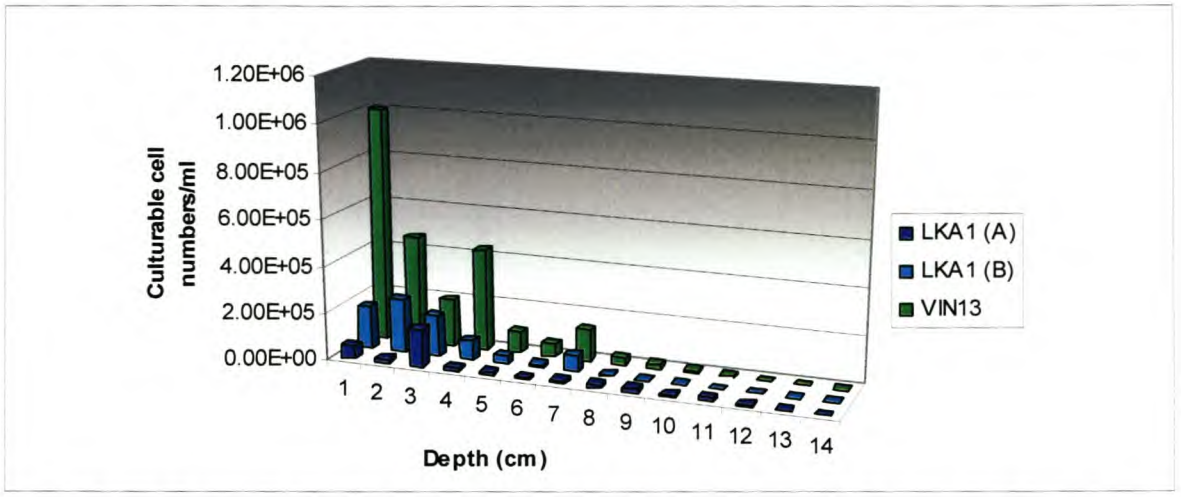
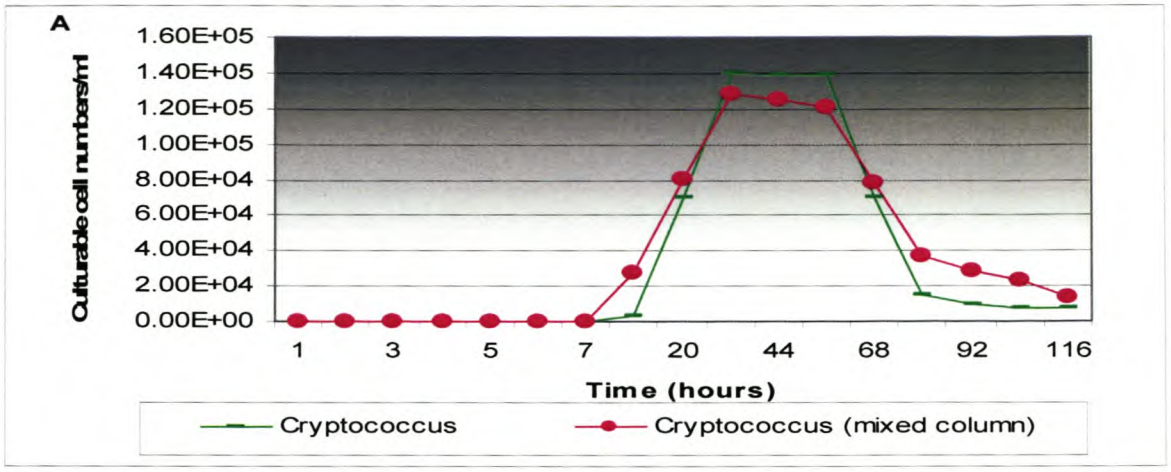
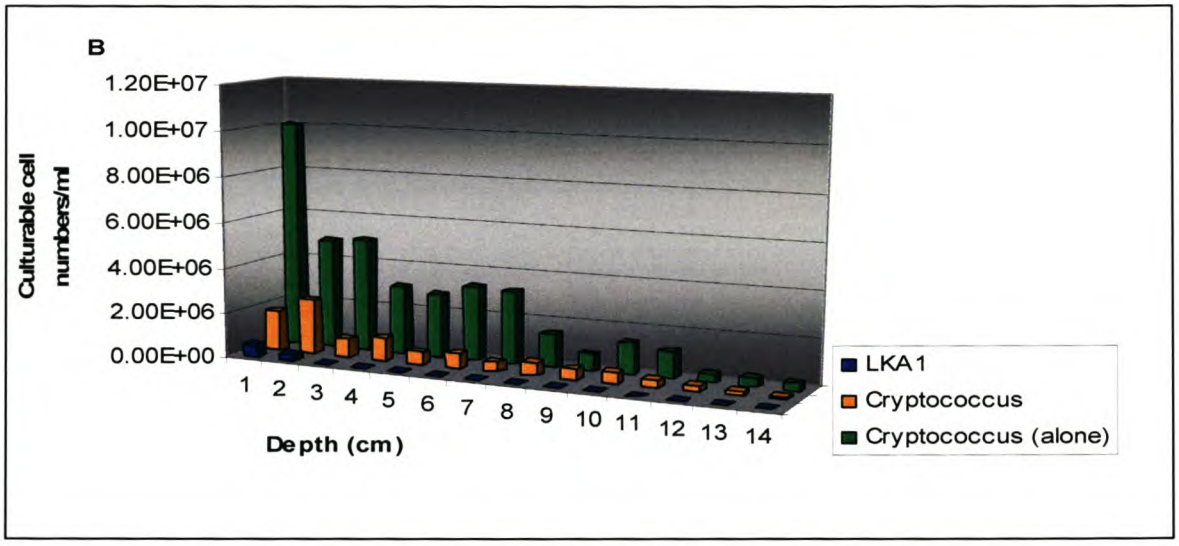


Fig. 5.2 Distribution of VIN13 and LKA1 through sand columns, showing that the cells were attenuated in the sand. A constant flow of the irrigation medium was maintained through the column (0.125 PV/h). No yeast cells were detected in the effluent, even after extended periods of flow (≤ 36 PV's). VIN13 represents the mean values of a column that was run in duplicate for 36 PV's (290 h), while LKA1 (A) and LKA1 (B) were run for 17 PV's (137 h) and 9 PV's (76 h), respectively.

the sand. Dissection of the columns and subsequent plating revealed that *Cryptococcus* cells were distributed along the length of the column, while LKA1 cells were detected only in the first 2 cm (Fig. 5.3B). The higher numbers of *Cryptococcus* in the first 2 cm demonstrate the competitive advantage that *Cryptococcus* has on LKA1 in soil. This observation may be because soil is the natural habitat for *C. laurentii,* but also because of the known ability of *Cryptococcus* spp. to produce antimicrobial substances that could inhibit the *Saccharomyces* strains. Support for this is the observation that, when introduced in pure culture, LKA1 migrated as far as 12 cm through the column, compared to 2 cm when co-introduced with *Cryptococcus.* Considering the possibility that *S. cerevisiae* originated from the hybridisation of other *Saccharomyces* species in man-made environments, it is likely that *Cryptococcus* may have evolved better strategies for distribution and proliferation in soil than *Saccharomyces*. This is especially of interest when it is taken into account that the two yeasts have cells of similar size. Overall, there was not a difference in the mobility of the genetically modified and unmodified *Saccharomyces* strains in saturated sand.

Similar to *Cryptococcus,* the bacterium *D. fermentens* could be detected in the column effluent after 1 PV, and when the columns were dissected at the end of each experiment, cells were found distributed along the full length of the columns (Fig. 5.3C). When mass balances were performed, the total numbers of cells detected in the columns inoculated with *Cryptococcus* and *D. fermentens* were higher than the respective cell numbers introduced to the respective columns.





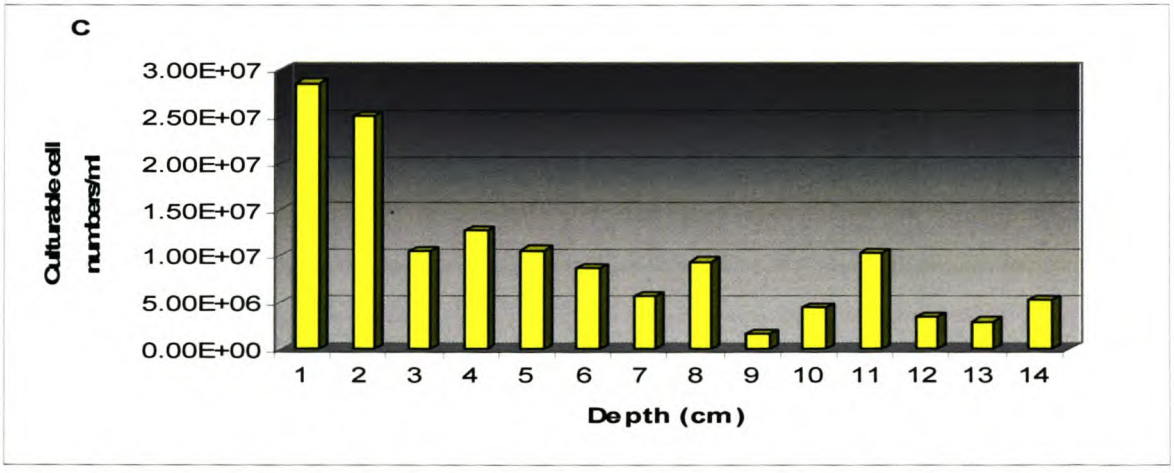


Fig. 5.3 Mobility and survival of the soil yeast *Cryptococcus* in saturated sand. A) In contrast with the *Saccharomyces* strains, *Cryptococcus* broke through after 1 PV (9 h). The presence of LKA1 did not affect the behaviour of *Cryptococcus*. B) Distribution of *Cryptococcus* and LKA1 through sand columns after 14 PV's (108 h), showing that similar to its breakthrough profile (A) the presence of LKA1 did not affect the distribution of *Cryptococcus*. In contrast to the *Saccharomyces* strains, *Cryptococcus* was found throughout the length of the column either when co-inoculated with LKA1 or inoculated on its own, supporting the breakthrough data shown in (A). (C) Similar to *Cryptococcus*, *D. fermentens* was distributed throughout the length of the column and broke through after 1 PV (results not shown). Column was run for 19 PV's (145 h).

A possible explanation for this observation is that both these organisms are well adapted to proliferate under oligotrophic conditions; therefore the Ringers solution and impurities associated with sand were sufficient to support prototrophic growth by these organisms.

5.3.2 THE SURVIVAL OF GENETICALLY MODIFIED LKA1 IN SOIL OVER TIME

The modified LKA1, and unmodified VIN13 *Saccharomyces* strains were detected in the soil in comparable numbers two weeks after inoculation (Table 5.1). However, after longer periods of incubation (7≤12 weeks) the organisms were either not detected, or were present in numbers that were too low for enumeration. This result is in agreement with a number of studies that have investigated the environmental fate of bacterial inoculants in soils (Van Elsas *et al.*, 1998). Bacteria introduced to soils generally responded with a decline in population density, which often resulted in their persistence at low densities or complete loss. Several biotic and abiotic factors can affect this behaviour and have been reviewed by Van Veen *et al.* (1997) and Stotzky (1980; 1995). Following the failure to detect introduced yeasts in the present study, after prolonged incubation in the soil, it was decided to use an enrichment strategy, primarily to assess whether there were any surviving cells in the soil. Both strains could be enriched from the soil after 14 weeks when Colombard must was used as enrichment medium. However, after 16 weeks, no statistically meaningful data could be obtained and therefore 1% YM medium was used for enrichment on week 18. These results indicate that cells of both the modified and unmodified *Saccharomyces* strains remained viable in vineyard soil over an extended period of time, although the majority of cells lost their culturability.

Table 5.1 Detection of VIN13 and LKA1 in vineyard soil over time.

	VIN13	LKA1	Enrichment
Week 2	3.75E+04 CFU/ml	3.15E+04 CFU/ml	None
Week 7	S/I*	S/I	None
Week 10	S/I	S/I	None
Week 12	N/D*	N/D	None
Week 14	(+)	(+)	Colombard must
Week 16	(-)	(+)	Colombard must
Week 18	(+)	(+)	1% YM medium

S/I - numbers too low to be statistically meaningful; N/D – not detected; + = presence indicated by enrichment method; - = not detected, despite of enrichment method

5.3.3 BIOFILM FORMATION

The data presented in Fig. 5.2 and 5.3 suggested that the test yeast strains attached to sand grains during the column studies. Therefore, the subsequent experiments focused on biofilm formation by these organisms. Initially, this involved the packing of flow cells with the same sand, and irrigating with Ringers solution, similar to the

column experiments. Pure cultures of *Cryptococcus,* VIN13 and LKA1 were grown overnight and inoculated (at 1.78E+08 and 6.90±0.2E+07 CFU/ml, respectively) into separate flow cell chambers, while *Cryptococcus* was inoculated together with LKA1 at original cell counts of 6.40E+06 and 8.00E+07 CFU/ml, respectively. Both VIN13 and LKA1 attached to the sand grains in pure culture, but they were notably less extensive than the biofilms formed by *Cryptococcus* (data not shown). When co-cultured, LKA1 could not easily be detected among the *Cryptococcus* cells although it was present in the flow cell effluent (data not shown). Furthermore, there was no indication that LKA1 had a negative effect on the biofilm formation ability of *Cryptococcus*.

As expected, when 1% YM medium was used as irrigation medium, thicker biofilms formed (Fig. 5.4). Due to the uneven nature of the attachment surface of sand grains, and the inherent difficulty to accurately determine the extent of biofilm formation, the latter was not quantified. However, replicate experiments strongly suggested that the biofilms formed by *Cryptococcus* were more expansive than those formed by the two *Saccharomyces* strains when cultivated under the same conditions. It was further observed that the cell distribution of LKA1 biofilms were less dense than those formed by VIN13. Routine staining of VIN13, LKA1 and *Cryptococcus* biofilms with Calcofluor™ White revealed the formation of extracellular polymeric substances (EPS) (Fig. 5.4A, 5.4B and 5.4C). EPS formation by VIN13 and LKA1 was similar, but notably denser for *Cryptococcus*. At the end of the experiments, flow was stopped and the biofilms were stained with FUN-1 ™ to set an indication of the viability of the attached cells. Overall, the biofilms of both VIN13 and LKA1 contained a high percentage of viable cells, suggesting that these strains are capable of a biofilm mode of existence (Fig. 5.4D, 5.4E and 5.4F). SEM observations of the sand grains confirmed that both these strains could form biofilms on sand (Fig. 5.5A to 5.5D). Similar to the images obtained by epifluorescence microscopy, the SEM images show dense packaging of cells and EPS formation. *Cryptococcus* formed notably denser biofilm and thicker layers of EPS formation in comparison to VIN13 and LKA1 (Fig. 5.5E and 5.5F).

One of the parameters known to influence the success of microbial adhesion is the substratum roughness, where irregular surfaces may result in increased bacterial transport and attachment. For instance, it was observed that cells attach mainly on the flat regions of a surface rather than within crevices (Baker, 1984). In contrast, others indicated that attached bacterial cells were preferentially distributed within crevices on rough surfaces (Notermans *et al.*, 1991).

Typically, active biofilms release cells into the planktonic phase. In this study, relatively high numbers of cells were found in the flow cell effluent, as shown in Fig. 5.6. Interestingly, despite the fact that *Cryptococcus* formed more extensive biofilms than the two *Saccharomyces* strains, the numbers of culturable cells in the effluent were mostly similar, suggesting that the genetic modification did not improve the reproductive success of LKA1 when compared to the unmodified VIN13. Overall,

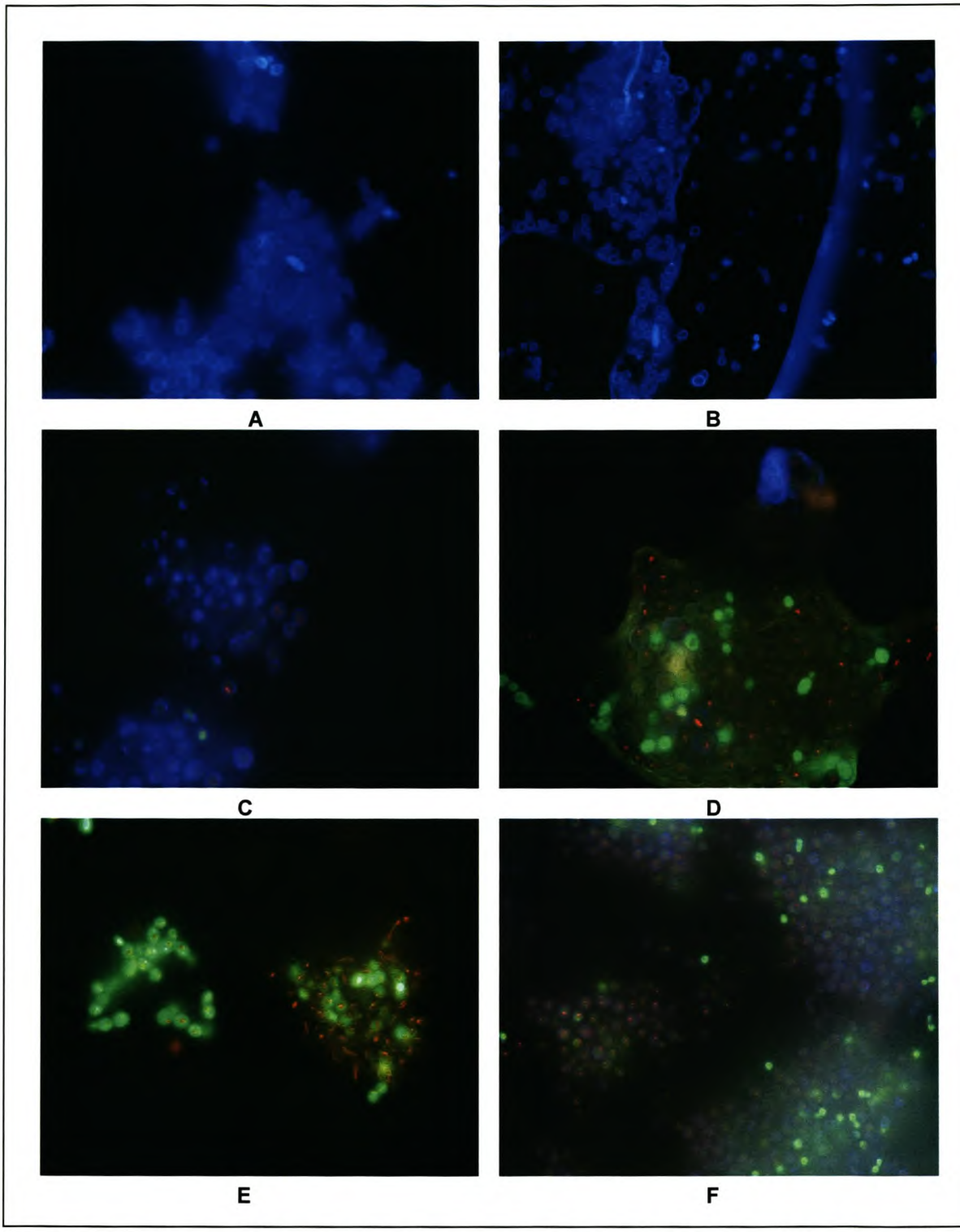


Fig. 5.4 Photomicrographs to demonstrate attachment of VIN13 (A and D), LKA1 (B and E) and *Cryptococcus* (C and F) to sand grains. Attachment of the cells during oligotrophic conditions such as when no exogenous carbon was provided in the irrigation medium (Ringer solution), without subsequent growth and detachment may provide a possible explanation for the observations made during the column experiments. When biofilms irrigated with 1% YM medium were stained with Calcofluor™ White, the presence of copious amounts of EPS could be observed on day four (A, B and C). Cells stained red with FUN-1™ is an indication of viable cells, while green cells represent dead cells (D, E and F). (D) and (F) were taken on day seven and (E) on day 12. (C) and (F) were stained with Calcofluor™ White and FUN-1™.

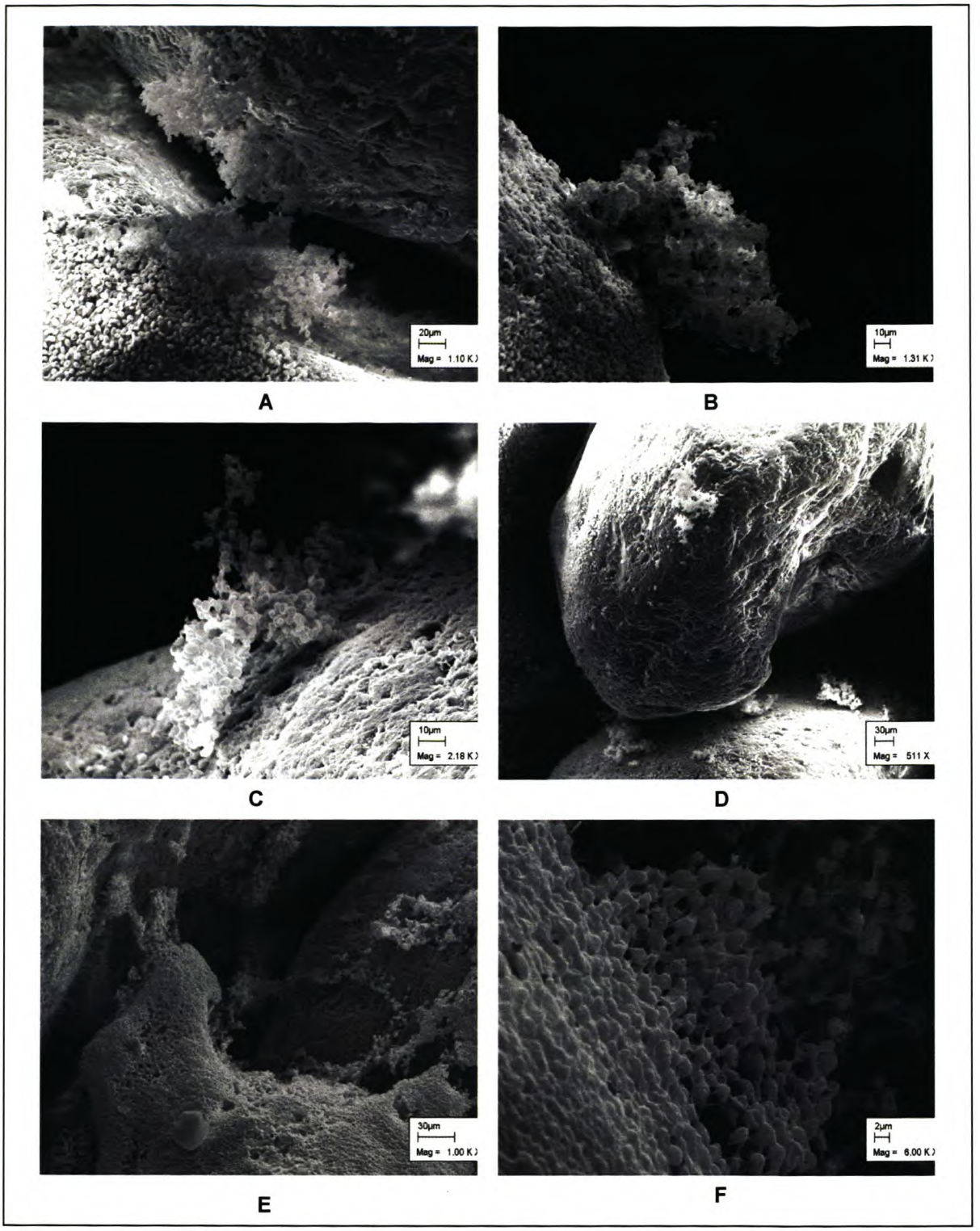


Fig. 5.5 SEM micrographs show attachment of VIN13 (A and B), LKA1 (C and D) and *Cryptococcus* (E and F) to sand at a higher magnification. Biofilms were developed for 17 days in 1% YM medium.

it was observed that there were either no significant differences in the biofilm density of VIN13 and LKA1 on sand particles or LKA1 formed less dense biofilms than VIN13, as was the case with 1% YM medium as flow medium.

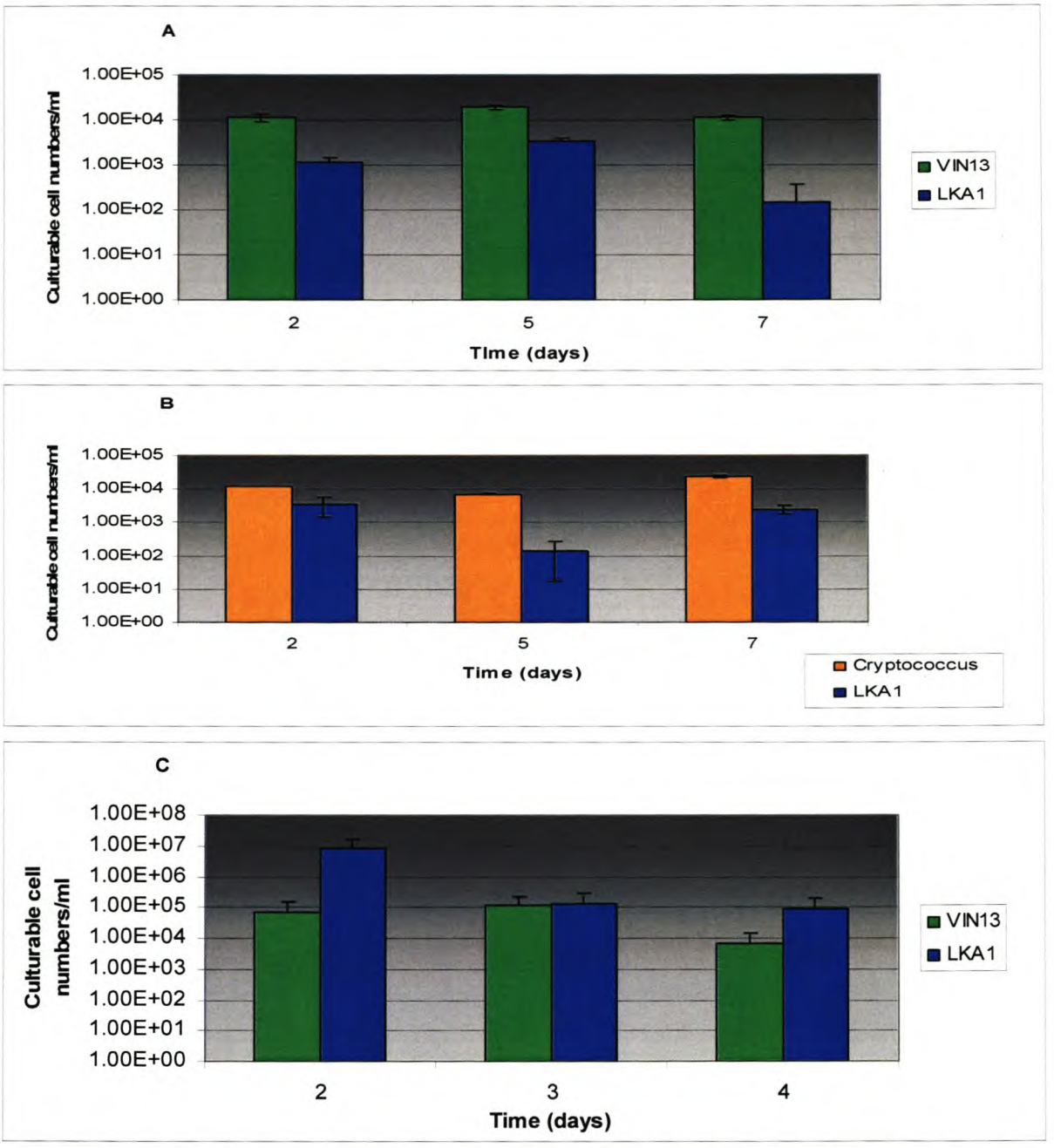


Fig. 5.6 Numbers of culturable cells in the effluent of flow cells filled with sand. The detection of pure cultures of VIN13 and LKA1 in flow cells irrigated with Ringers solution (A) and 1% YM medium (C). The detection of *Cryptococcus* and LKA1 co-inoculated in flow cells irrigated with Ringers solution (B).

Conventional flow cells were also utilised to compare the biofilm formation potential of LKA1 with the unmodified strain VIN13, and *Cryptococcus*. Similar to observations made in the sand-filled flow cells, it was found that biofilms formed by *Cryptococcus* were notably denser than those formed by *Saccharomyces* strains (Fig. 5.7). This difference was observed for the duration of the flow cell experiments, which lasted up to eight days. Generally, there was not a significant difference in the numbers of culturable cells in the respective flow cell effluents (Fig. 5.8A).

Data on the number of cells detected in biofilm effluent showed that both VIN13 and LKA1 were stabilised in the biofilm and that the numbers in their respective

effluents were similar when grown on 1% YM medium or 10% AWE (Fig. 5.8A and 5.8B).

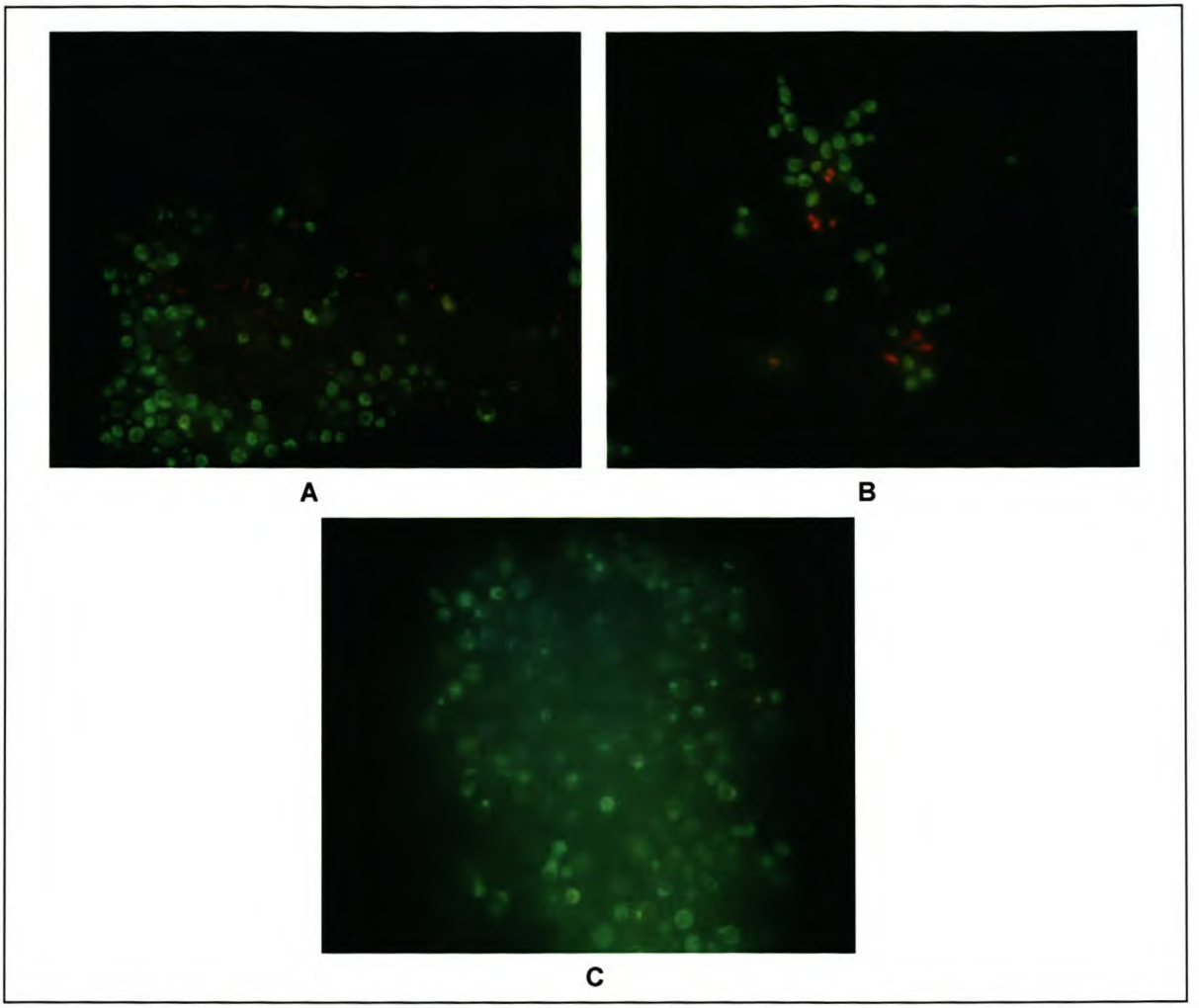


Fig. 5.7 Photomicrographs demonstrating biofilm formation of VIN13 (A), LKA1 (B) and *Cryptococcus* (C) during conventional flow cells. Biofilms were irrigated with 1% YM medium for eight days and stained with FUN-1™. (C) was also previously stained with Calcofluor™ White.

All three test organisms, VIN13, LKA1 and *Cryptococcus* showed the ability to form biofilms when monitored with an optical large area photometer (Fig. 5.9). From the beginning, biofilms formed by *Cryptococcus* and VIN13 were relatively stable, since the optical density readings only varied between 4600 and 4680. Biofilm formation for LKA1 showed more fluctuation and varied between optical density readings of 4200 and 4700. However, after a period of four days, the biofilms of all three test organisms reached a stable state. We do not have an explanation for the difference between VIN13 and LKA1 during the early stages. In general, there is relatively little information on the reproducibility of biofilm structure. Others have shown variability in bacterial biofilm profiles when pure cultures were cultivated under carefully controlled experimental conditions (Bester, Joubert, Garny and Wolfaardt, unpublished data; Lewandowski *et al.,* 2003). It is probable that biofilm function is maintained by a dynamic attachment-detachment behaviour, which may be amplified by experimental conditions.

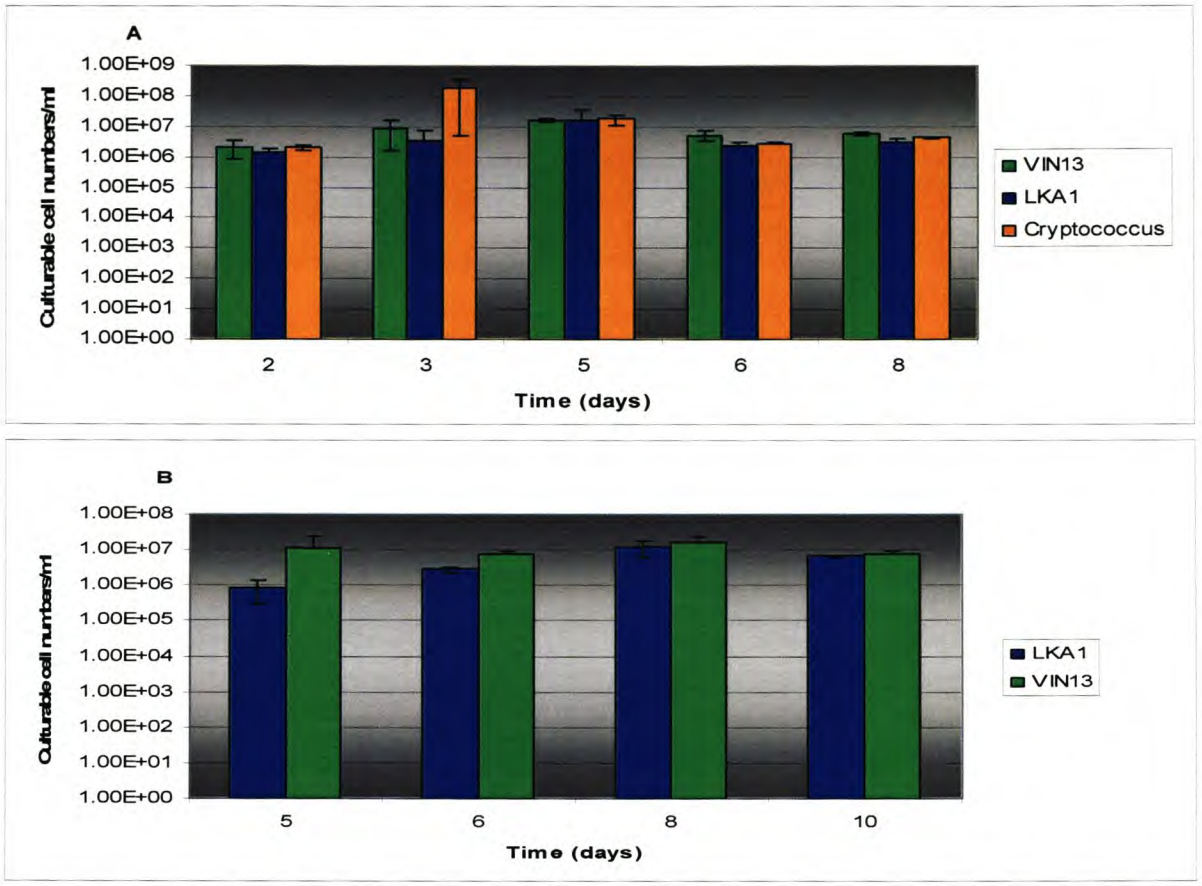


Fig. 5.8 Numbers of culturable VIN13 and LKA1 cells in the effluent of conventional flow cells irrigated with (A) 1% YM medium or (B) 10% AWE.

It has been reported that haploid cells adhere better to plastic surfaces than diploid cells (Reynolds and Fink, 2001). This is possibly because there is reduced expression of *FLO11* in diploids compared with haploids since it has been shown that *FLO11* expression decreases as ploidy increases (Galitski *et al.*, 1999). This could have implications in terms of the ability of laboratory strains versus industrial yeast strains to form biofilms if ploidy plays a large role. The industrial strains used in this study (VIN13 and LKA1) are diploid and it is possible that the environmental yeast, *C. laurentii,* is polyploid. It might be speculated that the difference in ploidy might have an influence on the ability of the yeasts to form biofilms. However, little information is available in the literature on this and it might be that other factors together with ploidy play an important role in enabling environmental yeasts to survive in biofilm mode.

5.3.4 THE INFLUENCE OF THE ADDITION OF LKA1 ON A SETTLED BIOFILM COMMUNITY

When a biofilm community was established that consisted of different yeast (*S. pombe, K. apiculata, S. ludwigii*) and bacteria, it was evident that *S. pombe* was not successful. No traces of *S. pombe* could be found in effluent collected on day

three and it can be assumed that all *S. pombe* cells were washed out by that time. WL nutrient agar was used to differentiate between the different yeast genera in the

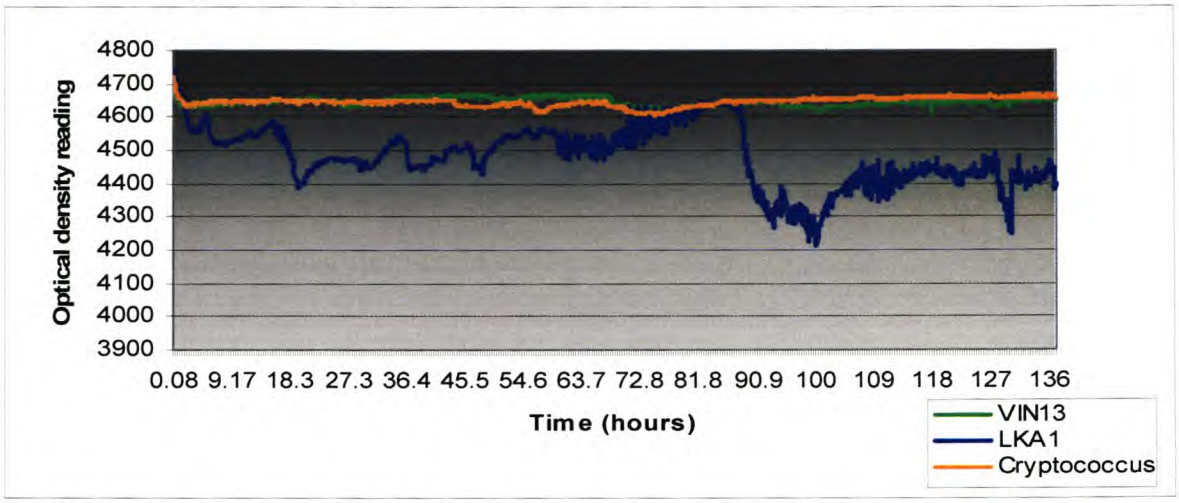


Fig. 5.9 The formation of biofilms by VIN13, LKA1 and *Cryptococcus* as shown through an optical large area photometer irrigated with 1% YM medium.

effluent, since each genus displays unique colony morphology when grown on this medium. *K. apiculata* was shown to readily form biofilms, and light microscopy images demonstrated that *K. apiculata* prevailed in high numbers in the biofilm community (results not shown). However, light microscopy also revealed that LKA1 did not incorporate well into a stable biofilm community (results not shown). Very few LKA1 cells could be visualised in the mixed-community biofilms, although LKA1 was still detected at a concentration of 3.00E+04 CFU/ml in effluent collected on day ten (Fig. 5.10). The numbers of the bacterial members of the biofilm community in the effluent remained stable over time, suggesting that the bacteria played a role in maintaining biofilm integrity, a function that they probably also have in nature. In contrast, the numbers of *K. apiculata* and *S. ludwigii* cells in the effluent showed notable fluctuations. Reasons for the difference in biofilm formation by these yeast

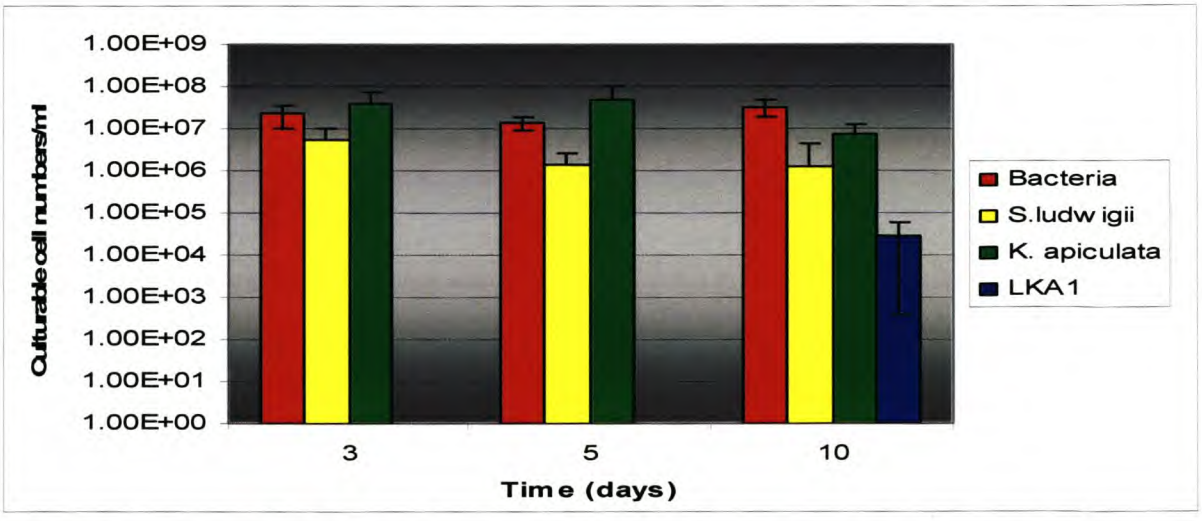


Fig. 5.10 Detection of the different community members together with LKA1 in the effluent of a conventional flow cell irrigated with 10% AWE.

species can only be speculative, since the field of yeast biofilms is still in its infancy. Overall, the addition of LKA1 to the stabilised biofilm communities did not disrupt the biofilm, since bacterial cells, *K. apiculata* and *S. ludwigii* could still be detected in significant numbers five days after the addition of LKA1.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

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6.1 CONCLUDING REMARKS AND OTHER PERSPECTIVES

We are reminded in our daily lives of the contributions of gene technology that were barely foreseen decades earlier. Many household goods we enjoy today are the fruits of biotechnological research. One of the biggest challenges of the wine industry is to show value and benefit from what can be achieved when innovators look over the horizon and see a future that awaits the consumer.

Consumers perceive wine as a harmonious blend of nature, art and science. The challenge for winemakers is to produce wines that are competitive in quality and price, and meet consumers' expectations for an enjoyable beverage that is healthful, produced in an environmentally-sensitive manner and still measures up to the traditional mystique of wine while making a profit along the production chain (Pretorius, 2005). While providing the grapegrower and the winemaker with the means to meet the challenges of coming decades, the potential afforded by genetically modified (GM) grapevine varieties and yeast starter strains, invites tension between tradition and innovation. Success in today's "demand-chain" environment (as opposed to the outdated "supply-chain" approach) means the wine industry must keep abreast of the application of new technologies if it is to meet the challenges presented by consumers and innovative competitors. Pacesetting grapegrowers and winemakers see it as a minimum requirement that they accurately measure and meet consumer preferences for style and quality and maintain the smart application of efficient practices along the supply chain, while at the same time leaving a minimal environmental footprint.

There is a multitude of complex and interconnected agronomic, business, regulatory and social obstacles blocking commercial availability of transgenic grapes and GM wine yeasts (Pretorius, 2003). Because of this, the commercial uptake of GM technology will not depend solely on science providing the wine industry with the capabilities to move ahead.

In this debate, it is my contention that the positioning of genetically modified organisms (GMOs) in general as either good or bad is unproductive. What is needed, rather, is scientifically sound information regarding potential risks associated with GM grape varieties and wine yeast strains. We will do well to recall the old saying: "one experiment might be more valuable than 100 so-called expert opinions" (Pretorius and Høj, 2005). Against this background the present study was initiated to investigate the potential environmental risks associated with GM wine yeasts.

The survival and prevalence of three GM strains of an industrial yeast, *Saccharomyces cerevisiae* VIN13, were monitored within a vineyard microbial community. To accomplish this, three blocks of grapevines were inoculated with

different combinations of GM yeasts over a period of two years within a secluded glasshouse.

One year (1999) was devoted to the establishment of the basal level of wild yeasts present on the leaves and berries. Wild yeasts were infrequently isolated from berries, but *Rhodotorula, Yarrowia lipolytica, Saccharomycodes ludwigii, Kluyveromyces lactis, Pichia* spp. and *Candida* spp were often detected on leaves. The *Candida* and *Pichia* species isolated from leaves were *C. parapsilosis, C. lambica, C. pulcherrima, P. guilliermondii* and *P. kluyverii.*

With few exceptions, the same yeasts were isolated the following year (2000) from grapevines in an untreated block (control block). *Y. lipolytica, Aureobasidium, Pichia* and *Candida* spp. were frequently detected on the leaves, as well as on the berries. Specifically, *C. pulcherrima* was isolated most often from the leaves and *C. parapsilosis* and *C. lyxosophila* from the berries, while *P. guilliermondii* and *P. anomala* were often detected on both the leaves and berries. *Pichia* spp. were consistently isolated throughout the season, while *Candida* spp. were mostly observed during August and September 2000. Yeasts isolated from the leaves were spread throughout the season each year. The average concentration of yeasts isolated from grapevines, however, were higher in 1999 than in 2000.

Of wild yeasts isolated from the grapevines in all treated blocks in 2000, *Rhodotorula, Y. lipolytica, Hanseniaspora, Aureobasidium, Cryptococcus, Debaryomyces, Pichia* and *Candida* spp. were most prevalent. Most of these yeasts were also frequently isolated from grapevines in the control block, indicating there was no substantial change in the variety of wild yeasts detected due to the introduction of VIN13 and GMY1 in the treated blocks.

Candida and *Pichia* spp. were among the dominant yeasts isolated from all the blocks in 2000. Across all the treated blocks, *Candida* spp. (specifically *C. parapsilosis* and *C. lyxosophila*) were isolated more often from berries than leaves. The variety and occurrence of *Candida* spp. among the four blocks in 2000 was variable and inconsistent, and therefore the introduction of VIN13 and GMY1 had no significant effect on the prevalence of *Candida* spp. While there was a tendency for more *Candida* spp. to be isolated from August 2000 onwards, *Pichia* spp. were present for most of the season. *P. guilliermondii* was frequently detected on leaves and berries from the treated blocks. The occurrence of *Pichia* spp. in the three treated blocks was remarkably similar, but differed from the control block. This suggests that there was a possible inoculation effect on the occurrence of *Pichia* spp. when VIN13 and GMY1 were inoculated on their own or together. However, there was no difference in the effect of the modified and the wild *Saccharomyces* on the occurrence of *Pichia* spp., which confirmed the null hypothesis that there is no difference between the behaviour of VIN13 and GMY1 on grapevines.

There were changes in the profile of wild yeasts over time. Interestingly, yeasts expected to be less prevalent on grapes, such as *Candida, Pichia, Rhodotorula* and *Cryptococcus*, were most frequently isolated, while supposedly more common

yeasts such as *Kloeckera* and *Hanseniaspora*, were seldom isolated. In line with other investigations (Martini *et al.*, 1996; Mortimer and Polsinelli, 1999), *S. cerevisiae* was seldom isolated from grapevines. The addition of the yeasts onto the grapevines had no significant effect on the reported occurrence of wild yeasts, since this observation was also made in the Control block. It can be concluded that the occurrence of wild yeasts on grapevines in a secluded glasshouse environment is notably different from the reported wild yeasts on normal grapevines. Although it is possible that factors such as fungal inhibitors, fungicides, yeast isolation methods and enrichment media might have had an influence on the yeast dynamics, it cannot be excluded that the microflora on grapes originating from a secluded glasshouse environment might be less diverse, at least with respect to yeast.

Inoculated VIN13 was not prevalent on the leaves, but most frequently isolated from the berries in block (VIN13) during September 2000 and 2001. After September of each year, VIN13 either declined in numbers (2001) or could not be isolated at all (2000).

In block (GMY), GMY1 was isolated during one sampling event from the leaves, but more often from the berries at relatively high numbers. Where VIN13 and GMY1 were co-inoculated in block (VIN13+GMY1), both were isolated from the berries at several sampling events. Although at relatively low numbers, they were detected approximately in the same ratio at which they were inoculated (1:1).

Inoculated GMY1 and GMY2 were isolated only from berries in block (GMY1+GMY2) and with relatively high counts. At first, GMY1 was detected in numbers lower than for GMY2, after which GMY1 and GMY2 were isolated in nearly equal numbers.

The GM yeasts inoculated in block (GMY1+GMY2+GMY3) were mostly isolated from the berries. For both months of sampling, the pattern of GM yeast detection was in the order of GMY2>GMY1>GMY3. A decline in GM yeast concentrations could also be detected after the second month of isolation. With a few exceptions, the overall detection of GM yeasts was numerically limited.

No GM yeasts were detected on pieces of vine branches and bark sampled during winter. Only during three sampling events were GM yeasts isolated, and in low numbers, from soil sampled from blocks (GMY1+GMY2) and (GMY1+GMY2+GMY3). GMY2 was most often isolated from the soil, while no GMY3 could be detected.

Data on the spontaneous fermentation of must originating from berries harvested from the different blocks indicated that the inoculated GM yeasts survived on the berries. Since no significant differences were observed between the fermentations of berries that were inoculated with the modified and unmodified strains of VIN13, the natural fermenting ability of the unmodified strain, VIN13, was conserved in the recombinant strains, GMY1, GMY2 and GMY3.

Statistical analysis of the spontaneous fermentation of berries from block (VIN13+GMY1) in 2000 and (GMY1+GMY2) in 2001 showed that for each year in

only one instance (berries harvested in early November), did GMY1 outperform VIN13 and GMY2, respectively, in terms of cell numbers. The data further suggested that the recombinant yeast strains did not have a competitive advantage. The observed differences in relative abundance during the early stages of fermentations might have been due to different cell loads on the berries, and these differences were eliminated with time in the absence of a competitive advantage by any of the strains.

The results indicate that it is probably safe for the environment to utilise the specific GM yeasts investigated in this study. There was a significant decline in the detection of all GM yeasts on the grapevines each year and it can be speculated whether these yeasts will eventually die off over time. Only long-term studies could reveal this. Although it was observed that the addition of the yeasts had an effect on the relative abundance of the naturally-occurring *Pichia* spp., the GM yeasts had no negative influence on the occurrence of other wild yeasts. Furthermore, the GM yeasts were seldom isolated from soil, and never from pieces of vine or branches.

Small-scale fermentations with a controlled, mixed yeast inoculum were performed to alleviate any possible effect of inoculum size and to allow assessment of the relative fitness of the GM strains when subjected to direct competition. Fermentations were performed with combinations of yeasts that were representative of the combination of yeasts inoculated onto the grapevines in the glasshouse. Although co-inoculated at a ratio of 1:1, there was a significant difference between the behaviour of VIN13 and GMY1 for the duration of the fermentation. GMY1 was outperformed by VIN13. It is tempting to speculate that GMY1 might have lost the expression of its α-amylase, as it possibly conveys no competitive advantage in a fermentation environment and becomes a metabolic burden for the yeast. This was, however, not confirmed by polymerase chain reaction (PCR) or Southern blot analysis.

Comparing fermentations of grapes sprayed with (VIN13+GMY1) with grapes sprayed with (GMY1+GMY2), it seems that GMY2 behaved in a similar way as VIN13 towards GMY1. GMY1 and GMY2 performed significantly differently from each other for the duration of fermentation, because GMY2 outperformed GMY1.

In the fermentation where GMY1, GMY2 and GMY3 were co-inoculated, GMY2 behaved differently to GMY1 and GMY3 for the duration of the fermentation, while the latter two yeasts displayed notably similar behaviour. GMY2 had a competitive advantage in the fermentation, possibly due to the fact that it expresses both an endo-β-xylanase and an endo-β-1,4-glucanase. Although GMY3 expresses a pectate lyase and a polygalacturonase and should have had, in theory, an advantage in a must environment, this yeast was detected in low numbers. It is possible that the expression of the combination of these two genes puts too much of a metabolic burden on the yeast in this specific must environment.

Spontaneous and controlled fermentations inoculated with the same combinations of yeast as were sprayed on the plants gave contradicting results.

Only one out of three spontaneous fermentations performed with berries from blocks (VIN13+GMY1) and (GMY1+GMY2) showed a significant difference in the relative abundance of the inoculated yeasts. Fermentations performed with berries harvested during early November of both years indicated that the specific composition of the must during that time of the season might have had an influence on the prevalence of the GM yeasts: there was a significant difference in the overall detection of yeasts in fermentations performed with equal, controlled inocula of combinations of (VIN13+GMY1) and (GMY1+GMY2). GMY1 was outperformed by VIN13 as well as GMY2 in the respective fermentations. GMY2 even proliferated when it was co-inoculated with the other GM yeasts, GMY1 and GMY3. It is possible that the type of must, as well as the dosage response of the inoculated glasshouse berries, could have had a significant influence on the detection of the GM yeasts.

In terms of their behaviour in fermentations, the utilisation of these GM yeasts in the industry could have some implications with regard to the expression of the specific transgenes in different types of must. It might be possible that certain GM yeasts will not perform equally well in all types of must.

The mobility of GMY1 and a typical soil yeast, *C. laurentii*, were compared in three types of microcosm: saturated sand columns, saturated sand flow cells and conventional aqueous flow cells. Each laboratory scale model was chosen to observe the mobility behaviour, survival and attachment to sand particles and glass.

In contrast to *C. laurentii*, neither VIN13 nor GMY1 showed a breakthrough of cells through the sand column, even after the passage of up to 36 pore volumes. Dissection of the columns after experiments showed that the yeast cells did survive, but were not mobile in the porous matrix. The lack of mobility of the yeast within the column suggested that the cells attached with high efficiency to the sand grains. It is expected that the rough texture of the sand grains aided in the attachment of yeast.

When the results for the unmodified and modified yeasts were compared, no significant difference in the mobility pattern of the two strains could be observed. Speculating on the implication of the release of this recombinant strain in nature, it might be expected that it will not act significantly differently to the unmodified strain in soil.

After a prolonged period of incubation (four to five months) in typical vineyard soil, neither VIN13 nor GMY1 could be detected. Only after enrichment with Colombard must and 1% YM medium was the yeast detected, suggesting that the yeast do survive in soil, but lose some culturability.

A saturated, sand-filled flow cell system enabled direct observation of attachment to sand granules and subsequent biofilm formation, while conventional flow cells allowed observation of biofilm formation in an aqueous environment. All three yeasts (VIN13, GMY1 and *Cryptococcus*) were shown to form stable biofilms on sand granules and glass. Microscopic examination of the attached cells revealed

that they were round and yeast-form, similar to the observations when yeast attached to plastic surfaces (Reynolds and Fink, 2001).

However, the attachment of VIN13 and GMY1 to sand particles and glass was less extensive than for *Cryptococcus*. Epifluorescent staining of biofilms confirmed that both VIN13 and GMY1 were capable of existing in a biofilm, since a high percentage of viable cells could be detected. Although less extensive than *Cryptococcus*, *Saccharomyces* yeasts were also shown to have notable extracellular polymeric substances extracellular polymeric substances (EPS) production.

Data on effluent numbers detected in flow cells for both *Saccharomyces* strains indicated that GMY1 had no advantage due to the genetic modification and had the same reproductive success than VIN13. Even with different irrigation media (1% YM medium and 10% Artificial Winery Effluent), these yeasts did not act differently to each other. Microscopic observations revealed these strains either have no difference in biofilm density or GMY1 was less dense than VIN13, as was the case with 1% YM as irrigation medium. When co-inoculated with *Cryptococcus*, GMY1 had no negative influence on the breakthrough of this other yeast through a sand column, or on its ability to form biofilms. Five days after introduction of GMY1 to a stable biofilm community on glass, it was microscopically observed that GMY1 did not successfully incorporate into the community. GMY1 did not disrupt the biofilm community either, since member cells could still be observed in the flow cell effluent.

These findings contribute positively to the cause of utilising GM yeasts in the wine industry. The assessment of GMY1 in these model systems have opened up the investigation of other GM yeast strains important for the wine, brewing and baking industry. If specific GM yeasts are scientifically shown to be harmless to the environment and other natural occurring microbes, progress can be made towards the acceptance of the use of these yeast strains in the industry.

Microbes show great variety in the extent and type of biofilms they form. Such variation complicates the study of microorganisms in natural settings. A further complicating factor is the difficulty to find a correlation between growth of an isolate in pure culture and growth in a mixed-species biofilm (Costerton *et al.*, 1995). Demonstration in this study of the ability of *Saccharomyces* to form stable biofilms exemplified the need to include this mode of growth when the environmental fate and effect of industrial, and especially GM yeasts, is considered. Such information is a requirement in the continuing debate on the merit of GMOs.

The fact that we have shown that *Saccharomyces* is able to form stable biofilms opens many possibilities of exploiting such models in clinical research on such things as yeast pathogenesis and antifungal therapy.

The assessment of the potential ecological effects from the use of GMOs is a formidable process. A basic understanding of the structure and function of the indigenous microbiota in an environment, as well as understanding what represents an ecologically significant change in the structure and function of that environment is important. Without it the assessment of ecological effects, besides pathogenicity or

toxicity that could arise from the release of GMOs into the environment, will remain a difficult task (Doyle *et al.,* 1995).

The assessment of the potential ecological effects of GMOs before their release into the environment is usually done in laboratories and glasshouses. Mesocosms and glasshouses probably simulate field conditions better than laboratory microcosms. However, mesocosms have limited use in validating laboratory studies, because they still present an environmentally regulated and contained construct of the natural world (Doyle, 1995). A future prospect for the GM yeasts used in this study is to verify the assessment of any ecological effects caused by them through field trials.

It is imperative that safeguards concerning ecological consequences of GMOs are constantly being developed. Importantly, the effect of GMOs must be assessed over extended periods of time, since not all ecological processes are manifested over short periods (Magnuson, 1990).

In future, it will be worthwhile to investigate the behaviour of modified *Saccharomyces* strains used in this study, in a mixed-species set-up more extensively. It would be also be advantageous for future research to target ecologically "riskier" organisms in similar settings.

Despite the many challenges that will have to be overcome during the next few years, it would be unwise to pretend that there will be no remarkably innovative developments and ideas that will be of great benefit to the winemaker and consumer. There is widespread consensus that most of the initial fears regarding GM foods and genetic engineering are largely unfounded. No respected scientific test has shown so far that any of the GM foods currently available on our shelves pose a health risk. There is a growing consensus that risk is primarily a function of the characteristics of the product and not necessarily from the use of genetic modification *per se,* there is a broad consensus among scientists that regardless of the way in which a microorganism has been modified, whether it be through traditional or modern methods, they respond to the same physical and biological laws. It is therefore most probable that no conceptual distinction exist between a yeast that has been modified through classical methods and one that has been modified through genetic engineering (Pretorius, 2000).

Although gene technology has already enormously contributed to our basic understanding of the biochemical and physiological processes in yeast during wine fermentations, it is unwise to entertain unrealistic expectations. Any creative advancement is also required to comply with the strict regulations that relate to the use of GMOs and to address the negative reaction of some consumer groups. Undoubtedly there is a huge potential benefit to the wine consumer and the industry in the application of genetic engineering. For these benefits to be realized though, the application should be done cautiously and systematically and with great consideration for the product's unique nature (Pretorius, 2000).

For total acceptance of transgenic food in the future the focus will have to be on products with clear advantages for the consumer. It is hard to imagine that a genetically engineered yeast which would enable the production of top quality wine with significantly decreased levels of sulphur dioxide would be rejected by both the wine producer and consumer (Pretorius, 2000).

The successful application of recombinant DNA technology in the wine industry will depend on whether commercial users of GM wine yeasts are assured that characteristics that are desirable have not been compromised, that legislation requirements are met, that the engineered strain will perform stable in practice and that suitable procedures are in place for the monitoring of the new yeast strains (Pretorius, 2000).

It will also be imperative to compile a database of all the possible genetically modifications and their respective detection strategies that could be applicable in the wine industry, whether it be modifications of grapevines or wine yeast.

Not all biotechnological applications have received the same level of attention and criticism. The field of animal and human health applications have either not attracted any significant level of public attention or have been received positively. It is the fields of agricultural applications and GM foods that have been on the receiving side of many anxieties and criticism. It is of my opinion that the eventual acceptance of GMOs in many fields of application that will be specifically beneficial for the consumer, will rely to a large extent on the acceptance of the array of GM plant varieties that are currently being developed and tested e.g. maize, cotton, wheat, soya etc. Many of the aforementioned (unmodified) plant varieties represent the primary needs of most consumers. The public acceptance of the variety of beneficial GM plant products could influence the acceptance of the application of other GMOs in fields of consumer interests e.g. GM microorganisms such as GM yeasts.

Conventional methodology was applied in this study for the detection and enumeration of yeasts. Application of a more sensitive detection technique e.g. PCR might have provided some additional insight into the occurrence of the GM yeasts. However, to accommodate an extra detection technique in this study, would require that the scale of sampling, and numbers of replicates, be minimized. Valuable insight into the longevity of GM yeasts on grapevines can be provided by the extension of a study of this nature. For various practical reasons this was not possible, but for more extensive studies on the environmental impact of GM yeast on grapevines, longer term studies would be imperative. Furthermore, the use of enrichment procedures and PCR in the detection of GM yeasts within the grapevine soil could have provided insightful data, especially considering the fact that some GM yeasts were detected several months after inoculation into a model consisting of typical vineyard soil.

Perhaps the strength of this study was in the innovative use of model systems to investigate the possible environmental impact of the GM yeasts. This was also an

entirely novel approach into the investigation of the formation of biofilms by yeasts. For future research into the environmental impact of GMY2 and GMY3 it will be imperative to investigate the behaviour of these yeasts in similar model set-ups. More extensive studies into the behaviour of these yeasts within biofilm communities will be insightful. Overall, the utilization of the approach followed here can be invaluable in the risk assessment of all GM yeasts.

Clearly, the final go-ahead for any GMO requires an extensive and well-planned risk assessment program. The investigation of the GM yeasts in this study under different scenarios is a good start. This study provides a foundation for gathering much-needed scientific and technical information to inform industry and regulating bodies. The outcome of this research is also intended to serve as a basis for information sharing with pubic interest groups.

Major challenges in biotechnology still remain. In future we must use our insight, technology, dedication and drive to solve the problems associated with "new" (e.g. AIDS) and established diseases (e.g. cancer and parasitic infection), antibiotic resistance, environmental pollution and accumulating urban, industrial and agricultural wastes. To accomplish this, a continued interaction between different disciplines, major support by governments and international agencies and a more understanding and supportive public will be needed (Demain, 2000).

An improved world that utilises biotechnology more fully will not be determined by science alone, but will be shaped by society's institutions to adapt, adopt and utilise technology in ways that will be commercially and socially beneficial (Phillips, 2002). Hopefully, progress will enable the GM debate to swing from one of being pro or con GM technology to one in which the debate is concerned with evaluation of individual technology packages' on a case-by case basis.

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