Industry allocated project number

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Indicate (X) client(s) to whom this final report is submitted. Replace any of these with other relevant clients if required.

FINAL REPORT
2013

Programme & Project Leader Information

	Research Organisation Programme leader	Project leader
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Project Information

Research Organisation Project number	IWBT W10/01
Project title	Developing a fast and reliable technique for detecting *Brettanomyces/Dekkera* sp. and investigating their response to the presence of sulphur dioxide

Fruit kind(s)			
Start date (mm/yyyy)	01/2010	**End date** (mm/yyyy)	12/2012

Project keywords	*Brettanomyces/Dekkera bruxellensis*, fast detection, response to SO_2 exposure, viable but not culturable

Approved by Research Organisation Programme leader (tick box) ☐

THIS REPORT MUST INCLUDE INFORMATION FROM THE **ENTIRE** PROJECT

Executive Summary

Give an executive summary of the total project.

Dekkera/Brettanomyces bruxellensis is a spoilage yeast species that occurs in red wine. It displays an amazing ability to survive under harsh conditions such as high concentrations of ethanol and sulphites, very low concentrations of sugar and nitrogen. It spoils wine by producing high levels of volatile phenols, acetic acid and other undesirable compounds. It is suspected that its ability to survive in wine is due to its capacity to enter into a viable but non culturable (VBNC) state, under which its metabolism is reduced to vital functions and it displays a temporary loss of division. *D. bruxellensis* is therefore difficult to detect using traditional microbiological techniques such as plating.
This project had two main objectives: optimising a fast and accurate technique to detect *D. bruxellensis*, including cells in a VBNC state and exploring the cellular mechanisms of response to SO_2 exposure. For the first objective, quantitative real-time PCR was fully optimised and yielded excellent results in terms of reproducibility, detection accuracy and rapidity of analysis. Reliable enumeration can be achieved within one day. The method is able to distinguish between dead cells and VBNC cells and the detection limit is 10 cells/mL. For the second objective, it was shown that, like in *Saccharomyces cerevisiae*, SO_2 tolerance is highly strain dependent. Passive intracellular accumulation and active efflux did not correlate with SO_2 tolerance, showing that other mechanisms play a role. Acetaldehyde was shown to be a marker of cellular stress peaking after the addition of SO_2 but decreasing as the metabolic acitivity resumes. The VBNC state was also investigated as a response to SO_2. Flow cytometry was shown to be a reliable method to detect entry into the VBNC state that could be used in the future to study the molecular mechanisms pertaining to this peculiar physiological state. The lack of available genetic data at the time of the experiments prevented from generated results at a molecular level, but D. bruxellensis' genome have now been fully sequenced and this will greatly facilitate all future research. In this context, the future work will focus on the VBNC state, its molecular mechanisms and its connection to survival under sulphite stress using transcriptomic, proteomic and metabolomic tools.

Problem identification and objectives

State the problem being addressed and the ultimate aim of the project.

Dekkera bruxellensis is recognised worldwide as a major microbiological threat for red wines. Its ability to survive after the completion of alcoholic fermentation, despite the presence of high concentrations of alcohol, sulphur dioxide and tannins amongst common inhibitors and the very low content of sugars is amazing. This species is resistant to high levels of sulphur dioxide, but the intracellular mechanisms behind this resistance are not known. Moreover, it is believed that sulphur dioxide is the compound forcing the cells to enter into a viable but not culturable state, making them even more resistant and likely to reappear at the least expected time during maturation or even in the bottles. Once again, the mechanisms behind the entry and the exit from the VBNC state are yet to be unravelled.
It is essential for the wine industry to accurately detect and enumerate *D. bruxellensis* cells in order to prevent spoilage and/or to confirm spoilage before taking curative measures. Because of the peculiar physiology of *D. bruxellensis* (and its ability to enter into VBNC state in particular), it is believed that classical microbiology techniques are likely to give false responses (i.e. no detection of *D. bruxellensis* while it is present) and consequently to minimize the spoilage risk. In this context, the implementation of quantitative real-time PCR should provide the most accurate response.
This study has therefore been subdivided into two main sections:
- Section 1: Investigating the intracellular mechanisms of the metabolic and physiological responses of these yeasts to the presence of sulphur dioxide

- Section 2: Implementing a fast and reliable technique for detecting *Dekkera* spp. in wine at the Institute for Wine Biotechnology, based on the recent results published in the literature

Workplan (materials and methods)

List trial sites, treatments, experimental layout and statistical detail, sampling detail, cold storage and examination stages and parameters.

Section 1: Investigating the impact of sulphur dioxide on *D. bruxellensis* at a cellular/molecular level

Milestone 1: Broad investigation of the mechanisms of resistance to SO_2 in *D. bruxellensis*
In all the tasks of this milestone, the behaviour of *D. bruxellensis* will be compared to that of *S. cerevisiae* (as a model yeast) and to that of *Zygosaccharomyces bailii* (as another wine spoilage yeast, known for its high resistance to sulphur dioxide).
Task 1: Various strains of *D. bruxellensis* (mostly from the IWBT culture collection) will be grown in a synthetic medium and the composition of the medium will be analysed after the addition of various concentrations of SO_2, especially with regard to molecular, free and total SO_2 as well as acetaldehyde concentrations.
Task 2: Following the addition of SO_2, the intracellular concentration of SO_2 will also be monitored
Task 3: investigating the presence of sulphite reductase activity
This task aims at determining whether *D. bruxellensis* possesses a sulphite reductase, able to reduce sulphur dioxide to hydrogen sulphide (H_2S).
Task 4: investigating the presence of sulphite oxidase activity
This task aims at determining whether *D. bruxellensis* possesses a sulphite oxidase, able to oxidise sulphur dioxide to sulfate (SO_4^{2-}).
Task 5: investigating the presence of a “sulphite pump”, similar to that encoded by the *SSU1* gene in *Saccharomyces cerevisiae*.
The presence of such an active transporter to guide the efflux of sulphur dioxide out of the cell could explain the high resistance of *D. bruxellensis* to high concentrations of sulphur dioxide.
Tasks 2, 3 and 5 will be monitored as described by Park and Bakalinsky (2000).

Milestone 2: Further investigation of resistance to sulphur dioxide
Depending on the result of task 5 of the previous milestone, an attempt to isolate and sequence the gene coding for the sulphite pump will be made. This task will be accomplished based on the sequence of the *S. cerevisiae SSU1* gene and related sequences in other yeasts.

Milestone 3: Investigating the molecular mechanisms of the entry into the viable but nonculturable state
In all the tasks of this milestone, the VBNC state will be monitored or verified by comparing plate counts and living cell counts, visualised by epifluorescence microscopy.
Task 1: The impact of various antimicrobial compounds, with a special focus on sulphur dioxide, of the culturability of *D. bruxellensis* will be investigated. Sudden addition of a high concentration will be compared with progressive addition of low concentrations of these antimicrobial compounds.
Compounds to be tested: sulphur dioxide, dimethyl dicarbonate, sorbic acid and benzoic acid.
Task 2: Transcriptomics study for investigating the molecular response to the addition of SO_2
D. bruxellensis will be cultivated and its total mRNA extracted after induction of the VBNC state (by addition of SO_2). As the genome of *D. bruxellensis* is not fully known and has not been annotated, it is not possible to do a microarray analysis on this total mRNA pool. Therefore, the analysis will be performed by high-throughput sequencing. The pool of mRNA will be compared with that extracted from cells cultivated in the same conditions but that have not been submitted to SO_2 addition. The aim of this task is to identify genes involved in resistance to SO_2 and to the entry into the VBNC state.
Whenever possible, the genes whose transcription is drastically modified (genes silenced or overexpressed) will be identified. Genes coding for sulphite reductase or a sulphite pump will be particularly looked for as well as genes involved in mitosis and cell morphology, size and integrity.

Milestone 4: Investigating the exit from the VBNC state
Task 1: modelling the exit from the VBNC state
The aim of this task is to establish and optimise a protocol for inducing the exit from the VBNC

state by command. As described in the literature, a decrease in the free-SO_2 concentration and the addition of O_2 in wine should induce an exit from the VBNC state. Addition of O_2 and/or decrease of the free-SO_2 concentration by addition of acetaldehyde or increase of the pH will be tested in order to establish a model of exit from the VBNC state.
Task 2: Investigating the intracellular mechanisms of the exit from the VBNC state. The molecular response of cells exiting a VBNC state will be investigated as described in Milestone 3.

Milestone 5: Investigating the metabolic activity of *D. bruxellensis* in a VBNC state
Various red wines will be spoiled with *D. bruxellensis* and the VBNC state will be induced by the addition of different concentrations of SO_2. The production of volatile phenols as well as other compounds such as molecular-SO_2, total-SO_2, acetic acid and acetaldehyde will be monitored during several weeks/months in order to quantify the metabolic activity of the cells in a VBNC state. The time and conditions met to exit from the VBNC will be noted. This will greatly help establish a model of entry into and exit from the VBNC state.

Section 2: Establishing a rapid and reliable technique to detect and enumerate *D. bruxellensis* in wine

Milestone 1: optimisation of the nucleic acid extraction technique in wine, with a specific focus on red wine
Task 1: optimisation of DNA extraction
Wine contains many inhibitors of enzymatic reactions, including PCR. During extraction using traditional techniques, various inhibitory compounds such as polysaccharides and polyphenols are precipitated together with the DNA. Using the product of such an extraction as a template for PCR either totally inhibits the PCR reaction, yielding no product or drastically decreases the PCR efficiency, rendering the cell enumeration inaccurate and irreproducible. Techniques have been described in the literature to purify extracted DNA, but they must be optimised or even reconsidered in our institute.
Task 2: DNA Amplification from dead cells or from free DNA
This task aims at determining whether the amplification of DNA from dead cells is possible and if so for how long after the death occurs.
Optimally, the quantification should be based on mRNA and not on DNA. However to date, no gene has been identified as being transcribed at a constant rate when the cells are in the VBNC state. It is therefore important to ensure that the DNA of dead cells is not amplified.
Therefore, should task 2 of milestone 3 in section 1 reveal genes whose transcription level seems not to be affected by entry into the VBNC state, qRT-PCR will be tried on the cDNA obtained after reverse-transcription of the total mRNA extracted from wine.

Milestone 2: Detection and enumeration of *D. bruxellensis* in wine
Task 1: Evaluating the use of primers already published in the literature (Phister and Mills, 2003; Delaherche *et al.*, 2004; Tessonnière *et al.*, 2009). Detection limits will be determined.
Task 2: Investigating other genes as potential target for qRT-PCR. Detection limits will be determined and compared with those obtained in task 1.
Task 3: Various red wines will be tested in order to establish whether wine composition (with regards to polyphenols concentration, pH, ethanol content, etc.) influences the detection limit. The methods of DNA extraction optimised in milestone 2 will be tested and finally selected in this milestone.

Milestone 3: Verifying the capability of qRT-PCR to detect cells in an active and culturable state as well as in a VBNC state.
Task 1: Verifying the detection of cells in active and culturable state
This task will be accomplished by spoiling wine with *D. bruxellensis* cells and comparing cell counts on plates and the results obtained by qRT-PCR.
Task 2: Verifying the detection of cells in VBNC state
This task will be accomplished by spoiling wine with *D. bruxellensis* cells and comparing cell counts obtained by epifluorescence, on plates and the results obtained by qRT-PCR.

Results and discussion

State results obtained and list any industry benefits. If applicable, include a short discussion covering ALL accumulated results from the start of the project. Limit it to essential information only.

Complete the following table

Section 1: Investigating the impact of sulphur dioxide on *D. bruxellensis* at a cellular/molecular level

Milestone	Target Date	Extension Date	Date Completed	Achievement
1. Broad investigation of the mechanisms of resistance to SO_2 in *D. bruxellensis*	2010		2011	a. An accurate and reliable assay to measure the concentration of SO_2 in small volumes has been set up. b. The SO_2 resistance of a few strains was determined: The amount of free SO_2 was quantified and compared in three different growth media in order to determine their SO_2-binding capacity. SO_2 resistance was tested on plates containing different amounts of molecular SO_2 (taking into account the SO_2-binding capacity of the medium as calculated above). SO_2 resistance was found to be highly strain dependent, with ethanol having a synergistic effect for most strains. Some strains were highly sensitive to SO_2, others were highly resistance in the absence of SO_2 but became sensitive when ethanol was added and others appeared moderately tolerant so SO_2 with or without the presence of ethanol. It must however be noted that the plate assays do not seem to be very reliable in terms of reproducibility; liquid assays should be envisaged in the future. c. A method to measure the rate of intracellular accumulation and extracellular efflux of SO_2 was set up. Three strains of *D. bruxellensis* were compared to 3 strains of *S. cerevisiae*. The results confirmed what was previously reported in literature for *S. cerevisiae* and *D. bruxellensis* followed the same trends. The results also showed that influx and efflux rates were strain dependent rather that species dependent and did not correlate well with the SO_2 resistance determined above. It was therefore concluded that SO_2 resistance is not a complex phenomenon that is not simply correlated with the rate at which a strain can efflux SO_2. d. Acetaldehyde, acetic acid, D-glucose and pyruvic acid concentrations were determined in response to the addition of SO_2 to the medium. The influence of SO_2 on the production of primary metabolites was determined in YPD over a short period of time (i.e. 48h), mimicking growth conditions and in synthetic wine over a much longer period of time (i.e. 32 days), mimicking wine ageing conditions. One strain of *D. bruxellensis* was compared to one strain of *S. cerevisiae*. The results confirmed that *D. bruxellensis*' overall metabolism is much slower than that of *S. cerevisiae*. Under growth conditions (i.e. in YPD medium) The concentrations of SO_2 used did not have a strong impact on *S. cerevisiae* apart from a slightly higher production of acetaldehyde. The results showed that a high concentration of molecular SO_2 (1 mg/L) slowed the consumption of glucose and the acetaldehyde and acetic acid concentrations strongly increased. Under wine ageing conditions, the effect of SO_2 was even more obvious and this effect was proportional to the amount of SO_2 added. At high SO_2 concentrations, the sugar metabolism stalled for a

				few days and the acetaldehyde concentration increased. However, as soon as the sugar metabolism resumed, the acetaldehyde concentration decreased. Acetaldehyde could therefore be considered as a marker of cellular stress as the acetaldehyde concentration produced was not enough to detoxify SO_2. These interesting results and in particular the role of acetaldehyde need to be further confirmed.
2. Further investigation of resistance to sulphur dioxide	2011		Not fully completed*	Several attempts to retrieve the *SSU1* gene by using degenerate primers failed. Meanwhile, the genome of *D. bruxellensis* was fully sequencing (Curtin et al 2012) and the *SSU1* gene was retrieved from the online database. The sequence identified as an *SSU1* gene is very different from that of other yeast species which would explain why it was not possible to amplify it using degenerate primers. In this context, its function still needs to be confirmed.
3. Investigating the molecular mechanisms of the entry into the viable but nonculturable state	2012		Not fully completed*	Trypan blue and methylene blue have been evaluated as markers of viability in an attempt to use them for visualizing the entry into the VBNC state. It was seen that they are not suitable as they do not consistently show cell death in *D. bruxellensis*. The use of flow cytometry was therefore attempted in collaboration with Prof Hervé Alexandre from the University of Burgundy, France. Fluorescein diacetate was the dye used to investigate the VBNC state. This dye fluoresces only in viable cells, and can be detected using a flow cytometer, as green fluorescence. Various concentrations of SO_2 were tested ranging from as low as 0.4 mg/L molecular SO_2 to as high as 2.0 mg/L SO_2. Four strains of *Brettanomyces bruxellensis* were tested in a Synthetic Wine Media, and from the results obtained, it was clear that tolerance to SO_2 concentration is highly strain dependent. Where the IWBT Y121 strain (isolated in South Africa) was more susceptible to lower SO_2 concentrations (0.8 mg/L) than the LO2E2 strain (isolated in France) (1.6mg/L). It was also noted that the percentage of ethanol played a role to the degree of tolerance or sensitivity to SO_2 which varied extensively between strains. For every strain tested, we were successful in inducing a VBNCs tate using SO2 as an inducer for at least 1% of the yeast population for short periods of time. Further optimisation is required to confirm the sustainability of this state over longer periods of time but flow cytometry appears to be a reliable tool to monitor the VBNC state.
4. Investigating the exit from the VBNC state	2012		Not fully completed*	Same as for Milestone 3
5. Investigating the metabolic activity of *D. bruxellensis* in a VBNC state	2012		Not fully completed*	This milestone was not fully completed. However, it was noticed that in the presence of high concentration of SO_2, the cells were still able to produce volatile phenols and the production of ethyl guaiacol was favoured in comparison to that of ethyl phenol. In future studies, it would be interesting to confirm whether this production was done by culturable or non-culturable cells.
5. Journal publication/s – final milestone	2012		2012	One review article published. Results obtained above require confirmation before publication can be envisaged.

*The reason for some of the milestones not being fully completed is mostly the lack of genetic data (for retrieving genes of interest) or optimised techniques (for studying the VBNC state) available when the project started. The study of the VBNC state using flow cytometry has started in collaboration with Prof Hervé Alexandre from the University of Burgundy, France. We are confident that the newly sequenced genome and the collaboration with Prof Alexandre will allow this project to yield interesting results as part of project IWBT W13/01.

Section 2: Establishing a rapid and reliable technique to detect and enumerate *D. bruxellensis* in wine

Milestone	Target Date	Extension Date	Date Completed	Achievement
1. Optimisation of the nucleic acid extraction technique in wine, with a specific focus on red wine	2011	n/a	2011	a) Three different genomic DNA extraction methods were tested for optimal DNA extraction from red wine inoculated with *D. bruxellensis* cells. The method that gave the best results in terms of reproducibility and DNA purity was that using the Wizard genomic DNA isolation kit (Promega). c) RNA extraction was method optimized from red wine inoculated with *D. bruxellensis* cells. The optimised method included the washing of the cell pellet with PVP to remove the polyphenolic compounds and the use of acid phenol, chloroform and a high salt buffer.
2. Detection and enumeration of *D. bruxellensis* in wine	2011	n/a	2011	a) Specificity of primers was determined Three primer sets published in literature as well as those used in the Brettanomytest Vineo test kit (BioRad) all targeting different genes were tested. All primer sets were highly specific to *Brettanomyces bruxellensis* and could not amplify the DNA or cDNA of other yeast species. b) Standard curve analysis was performed on DNA and cDNA. The primer pair that gave the best results in terms of PCR efficiency and linearity was that of the Brettanomytest Vineo test kit targeting the actin-encoding gene. It was also noted that the cDNA (obtained from the RNA extracted as explained above) gave better results than genomic DNA as PCR template. The Act1 and Act2 primers (from Brettanomytest Vineo test kit) were therefore selected for further analysis. Cell enumeration was then carried out using 3 different techniques (i.e. plating, epifluorescence microscopy and qPCR) in order to test the reliability of qPCR. All cell counts correlated very well.
3. Verifying the capability of qRT-PCR to detect cells in an active and culturable state as well as in a VBNC state	2012	n/a	2012	The VBNC state was induced by exposing a population of *D. bruxellensis* in wine to 0.8 mg/L molecular SO2 (the VBNC state was confirmed by comparing plating and epifluorescence). cDNA originating from mRNA extracted from the cells shows to be a better target than genomic DNA to detect VBNC cells. The use of DNA indeed overestimated the population, probably because the DNA of dead cells was also amplified.
5. Journal publication/s – final milestone	2012	n/a	2012	One scientific article published

Accumulated outputs

List ALL the outputs from the start of the project. The year of each output must also be indicated.

See below

Conclusions

Section 1: Investigating the impact of sulphur dioxide on *D. bruxellensis* at a cellular/molecular level

The cellular mechanisms of the response to SO_2 exposure in *D. bruxellensis* were investigated in comparison to those of *S. cerevisiae*. Overall, the results show that *D. bruxellensis* displays the same mechanisms as *S. cerevisiae*, especially with regards to intracellular accumulation, extracellular efflux. With regards to the impact of SO_2 on the production of primary metabolites, the same trends were observed, the main difference being the higher production of acetic acid by *D. bruxellensis*, probably due to the well-known Custer effect in this species. One interesting result is the accumulation of acetaldehyde following SO_2 exposure that could be linked to inhibition of glycolytic enzymes by SO_2. Acetaldehyde would be a marker of cellular stress, but this needs further confirmation. Furthermore, like in *S. cerevisiae*, tolerance to SO_2 is highly strain dependent in *D. bruxellensis*. The strong assets of *D. bruxellensis* are certainly its ability to survive in nutrient depleted matrices such as wine and its ability to enter into a VBNC state. This aspect was explored using flow cytometry but it needs further investigation to optimise the amount of SO_2 necessary to induce the VBNC state in different strains.
All follow-up studies will now be conducted as part of the on-going IWBT W 13/01 project. The genome of *D. bruxellensis* has now been sequenced and genetic data are available online. This will greatly aid the study of the VBNC state using a transcriptomic approach.

Section 2: Establishing a rapid and reliable technique to detect and enumerate *D. bruxellensis* in wine

As planned initially, a qPCR technique was optimised for the accurate and fast detection of *D. bruxellensis* in red wine. Protocols to extract yeast DNA and total RNA from red wine were optimised. Our study has then shown that mRNA as template gave a more accurate determination of population size when compared to epifluorescence and traditional plating for spiked samples as well as naturally contaminated wines. The overestimation of cell populations using DNA versus mRNA was also shown in the samples tested. Importantly the qPCR assay was able to detect *D. bruxellensis* in the VBNC state and accurately estimated the population present in this preliminary study.

Technology development, products and patents

Indicate the commercial potential of this project, eg. Intellectual property rights or commercial product(s)

Section 1:

The following techniques have been optimised:
- Colorimetric assay to rapidly and accurately measure free and total SO_2 in small volumes (only 50 µL medium required)
- Method to evaluate SO_2 tolerance on plates
- Method to quantify intracellular SO_2 accumulation and active efflux
- Method to quantify major metabolites to follow carbon flux (especially in response to stress such as sudden exposure to SO_2)
- Flow cytometry to evaluate entry into and exit from the VBNC state

Section 2:

- Optimisation of DNA and RNA extraction methods from red wine (with quality and quantity suitable for further application requiring pure and clean nucleic acids such as quantitative real-time PCR)
- Selection of primer set for specifically and accurately detecting and enumerating *D. bruxellensis* cells from wine using quantitative real-time PCR
- Optimisation of the quantitative real-time PCR using genomic DNA and cDNA isolated from wine

- The optimisation of this technique has good potential for providing service to the wine industry (rapid and accurate detection and enumeration of living *D. bruxellensis* cells in red wine)

Suggestions for technology transfer

List any suggestions you may have for technology transfer

- Information regarding *D. bruxellensis* and the latest discoveries on its biology and survival in wine (including those originating from this project) could be disseminated via publication of a popular article.
- Information regarding the techniques available for detection of *D. bruxellensis* in wine could also be the subject of a popular article.
- The qPCR method for fast and accurate detection of *D. bruxellensis* in wine that has been optimised in this project could be used as a service to the wine industry, but the IWBT does not currently have the capacity and structure to act as such a service provider.

Human resources development/training

Indicate the number and level (eg. MSc, PhD, post doc) of students/support personnel that were trained as well as their cost to industry through this project. Add in more lines if necessary.

Student level (BSc, MSc, PhD, Post doc)	Cost to Project
1. MSc (Edward Duckitt; graduated in March 2012)	R 0
2. Researcher (Dr Elize Willenburg)	R 0
3. MSc (Marli Louw; graduation planned in Dec 2012 or March 2013)	R 57 000
4.	
5.	

Publications (popular, press releases, semi-scientific, scientific)

Willenburg E. and Divol B. (2012) Quantitative PCR: An appropriate tool to detect viable but not culturable *Brettanomyces bruxellensis* in wine. International Journal of Food Microbiology. 160: 131-136.

Divol B., du Toit M. and Duckitt E. (2012) Surviving in the presence of sulphur dioxide: strategies developed by wine yeasts. Applied Microbiology and Biotechnology. 95:601-613.

Duckitt E.J. (2012) Investigating the impact of sulphur dioxide on *Brettanomyces bruxellensis* at a molecular and cellular level. MSc thesis, Stellenbosch University.

Presentations/papers delivered

Duckitt E.J., du Toit M. and Divol B. (Nov 2012) Investigating the response to sulphur dioxide exposure in *Brettanomyces bruxellensis*: a preliminary study. 34th congress of the South African Society of Enology and Viticulture, Groot Drakenstein, South Africa.

Total cost summary of the project

TOTAL COST IN REAL TERMS	COST	CFPA	DFTS	Deciduous	SATI	Winetech	THRIP	OTHER	TOTAL
YEAR 1	2010					100 000	50 000	0	150 000
YEAR 2	2011					110 000	55 000	0	165 000
YEAR 3	2012					178 200	89 100	0	267 300
YEAR 4						0	0	0	0
YEAR 5						0	0	0	0
TOTAL						**R 388 200**	**R 194 100**	**R 0**	**R 582 300**