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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

In silico approach to identification of a novel gene responsive to submergence stress in rice

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Submergence is one of the major constraints to rice production. Bioinformatics approach has been widely used to identify candidate genes on many biological aspects. In the present study, a novel gene involved in submergence stress in rice, *Os07g47670* was identified by *in silico* approach. The amino acid sequence of Os07g47670 is highly homologous to hypoxia-responsive family proteins. No disordered regions are found in the Os07g47670 protein. In the *Os07g47670* gene promoter, there are two ARE cis-regulatory elements, indicating that Os07g47670 is associated with submergence responsiveness. The *Os07g47670* transcript levels are higher in roots of one or two-week old plants than in other tissues. Without the *Sub1A* gene, the expression level of *Os07g47670* in M202 is low under submergence, ACC treatment, and normal condition. However, in the *Sub1A* genetic background, the *Os07g47670* transcript level is strongly induced during submergence, and peaked at day 1 during submergence. The mRNA level of *Os07g47670* in M202(Sub1A) was also significantly increased by ACC treatment. High expression level of *Os07g47670* is correlated with the existence of the *Sub1A* gene. *Os07g47670* shares similar expression patterns with *Sub1A, ADH1, SLR1,* and *SLRL1* and are co-induced under submergence. Thus, we have documented *Os07g47670* as a novel gene associated with submergence stress response. The identification of *Os07g47670* will facilitate the understanding of the molecular mechanism of submergence tolerance in rice.

Key words: Rice (*Oryza sativa* L.), *in silico* approach, submergence, waterlogging.

INTRODUCTION

Rice is one of the most important crops, as a staple food for more than half the world's population (Chen et al., 2009). However, rice is subject to various abiotic stresses, such as drought, submergence, high salinity, and low temperature, resulting in significant damage to rice. Among these abiotic stresses, submergence is increasingly becoming a major production constraint affecting about 15-20 million hectares of rice land in South and Southeast Asia and causing a loss of up to $ 1 billion every year (Xu et al., 2006). Rice has developed numerous strategies to

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Abbreviations: QTL, Quantitative trait locus; **ARE,** anaerobic response elements; **ACC,** 1-aminocycloprop-1-carboxylic acid; **NJ,** neighbor-joining; **ADH,** Alcohol dehydrogenase; **SLR1,** Slender rice-1; **SLRL1,** SLR1 like-1; **ERF,** ethylene response factors.

cope with submergence. Much progress has been made to understand their strategies conferring submergence tolerance (Fukao and Bailey-Serres, 2008; Perata and Voesenek, 2007). *SNORKEL1* and *SNORKEL2*, two ethylene response factors (ERF) genes isolated from deepwater rice, trigger the elongation of internodes under submergence. Thus, deepwater rice grows out of the surface of water, allows gas exchange with the atmosphere, and prevents drowning (Hattori et al., 2009). However, submergence tolerance in most *Oryza sativa* cultivars is conferred by *Sub1A-1* gene. The physiological and molecular base of *Sub1A-1* has been extensively studied. The role of *Sub1A-1* is to suppress the elongation of shoot during submergence, thus limiting anaerobic catabolism and leading to the preservation of carbohydrate reserves (Xu et al., 2006; Bailey-Serres et al., 2010).

So far, *SNORKEL1*, *SNORKEL2*, and *Sub1A-1* are three major genes that have been identified in conferring submergence tolerance. The molecular model of *Sub1A-1* has been described that represents a big step forward towards understanding the regulation of submergence tolerance in rice (Hattori et al., 2009; Xu et al., 2006). However, submergence tolerance is a complex quantitative trait. The mechanism of submergence tolerance in rice is still unclear. Therefore, it is necessary to identify new genes or QTLs involved in submergence tolerance. Recently, four new QTLs were identified using mapping population derived from two moderately tolerant varieties, IR72 and Madabaru. Some progeny have an even higher survival rate than the FR13A-derived tolerant control (IR40931). Four QTLs were identified on chromosomes 1, 2, 9, and 12. The QTL on chromosome 9 was Sub1A-1 (Septiningsih et al., 2012). Thirty-two putative QTLs associated with seedling vigor in rice under submergence were detected. Two QTLs with more than 10% contribution to the total phenotypic variance were verified for involvement in shoot length determination (Manangkil et al., 2013). Microarray has been widely used to measure mRNA levels of many genes in particular cells or tissues at once (Wang et al., 2005; Shimono et al., 2003). Using oligonucleotide microarray combined with suppression subtractive hybridization, a number of submergence -responsive genes in FR13A and Goda Heenati were identified. Under submergence, two genes exhibited an opposing expression pattern between FR13A and Goda Heenati, and 324 genes were regulated by submergence only in one genotype and unchanged in their expression in the other (Xiong et al., 2012).

As databases of gene expression data continue to grow, our understanding of gene function grows as well. In the present study, we utilized available microarray data, applied bioinformatics techniques, and identified a novel gene associated with submergence response. The study of this gene will help to understand the mechanism of submergence tolerance in rice and the breeding of submergence tolerance rice varieties.

MATERIALS AND METHODS

Retrieval of microarray data and its analysis

The microarray data of leaves of 14-day old M202 and M202 (sub1A) seedlings that were subject to submergence for 0, 1, 6 days, respectively, were retrieved from those available at NCBI database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41103). Microarray data were analyzed according to Jung et al. (2010).

Database search, sequence analysis, phylogenetic tree and heatmap construction

The NCBI database (http://www.ncbi.nlm.nih.gov) was mined to identify genes homologous to *Os07g47670.* The amino acid sequence of the Os07g47670 protein was used as a query sequence to search the databases using BLASTP. Multiple sequence alignment analysis was performed using ClustalX (Chenna et al., 2003; http://bioinformatics.ubc.ca/resources/tools/clustalx). Phylogenetic trees of the aligned protein sequences were constructed using MEGA version 5.0 (Tamura et al., 2007; http://www.megasoftware.net) via the neighbor-joining (NJ) method with the following parameters: Poisson correction, pairwise deletion, and bootstrap (1000 replicates; random seed).

Bioinformatic analysis

Bioinformatic analysis of the *Os07g47670* genes, such as the nucleotide and deduced amino acid sequences, composition, physical and chemical characterization, and conserved domain sequences, was performed using the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (http://cn.expasy.org). The solubility of the recombinant proteins when overexpressed in *Escherichia coli* was predicted using the statistical model from the University of Oklahoma (http://biotech.ou.edu) (Zhuang et al., 2008). The folding states of the protein were predicted using the FoldIndex program (http://bioportal.weizmann.ac.il) (Zhuang et al., 2008). The cis-regulatory elements in the *Os07g47670* promoter were predicted using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html).

RNA isolation and expression analysis

Total RNA was extracted from leaves of seedlings at different time points under submergence. Leaf, root, shoot, stem, and panicle at different growth stages were also chosen to extract total RNA. First strand cDNA was synthesized using SuperScript-II reverse transcriptase according to the manufacturer's instructions (Invitrogen). The *actin1* gene (Os03g50890.1) was used as an endogenous control to normalize the expression data (Table 1). The qRT-PCR primers specific for the *ADH1*, *Sub1A*, *SLR1*, and *SLRL1* genes are listed in Table 1. Real-time PCR was conducted using the SYBR real-time PCR kit (Takara Japan) with IQTM SYBR® Green Supermixture according to the manufacturer's instructions (Bio-Rad USA). The reaction conditions are as follows: 94°C for 1 min; followed by 40 cycles of 95°C for 10 s; 55°C for 10 s.

Various environmental stress and hormone treatments

All submergence, drought, and hormone treatments were replicated in at least 3 independent biological experiments. For GA and ACC treatment, 14-day old M202 and M202(sub1A) seedlings grown in

Table 1. Primers used in this study.

Primers	Sequence (5´-3´)	Usage
Actin1 forward	ACAGGTATTGTGTTGGACTCTGG	Real-time PCR
Actin1 reverse	AGTAACCACGCTCCGTCAGG	Real-time PCR
Sub1A forward	CGGCCTCATCACAATCGGAG	Real-time PCR
Sub1A reverse	ATGTCCATGTCCATATGTCGTCG	Real-time PCR
ADH1 forward	TGTTGGAGAGGGTGTGACTG	Real-time PCR
ADH1 reverse	GCCATCACCAATCATCACAC	Real-time PCR
SLR1 forward	GATCGTCACCGTGGTAGAGC	Real-time PCR
SLR1 reverse	GAGGGAATCGAACATGGTGG	Real-time PCR
SLRL1 forward	GGCGGCGACAATAACAACAACAGT	Real-time PCR
SLRL1 reverse	TACAAACACACGCTGCTACCATCC	Real-time PCR
Os07g47670 forward	ATGGCGGAGGAGAAGAGC	Real-time PCR
Os07g47670 reverse	GCAACCTGGCGTGGATGA	Real-time PCR

germination paper were transferred into mock (0.1% DMSO), GA (10 µM), or ACC (10 µM) solution, respectively, and treated for 6 h. Submergence treatment was carried out as described in Fukao and Bailey-Serres's report (Fukao and Bailey-Serres, 2008). Thirty five-day old M202 and M202(sub1A) were subject to drought stress for 0, 3, 5, 7 days in greenhouse, respectively. Drought treatment was carried out according to Xiong et al. (2014).

RESULTS

Expression of the *Os07g47670* gene under submergence treatment

The microarray data in M202 and M202(sub1A) under submergence stress were downloaded from the NCBI database, and analyzed. The expression level of the *Os07g47670* gene under submergence is shown in Supplementary Table 1. At normal conditions, the mRNA level of *Os07g47670* in M202 is approximately 6.0 fold higher than that in M202(Sub1A). However, under submergence, the mRNA level of *Os07g47670* in M202(sub1A) was strongly induced and peaked at day 1. On the contrary, the expression level of *Os07g47670* in M202 decreased under submergence. Consequently, under submergence for 1 day, the expression level of *Os07g47670* in M202(sub1A) increased 6.5 fold, and is approximately 5.0 fold of the level in M202 (Supplementary Table 1).

To verify the results of microarray, we performed quantitative real time RT-PCR (qRT-PCR) experiments. The qRT-PCR results show that the transcript level of *Os07g47670* in M202(Sub1A) was strongly induced during submergence from day 1 to day 6 and peaked at day 1. In parallel, the transcript level of *Os07g47670* in M202 was also increased during submergence. Most importantly, the *Os07g47670* level in M202(sub1A) is significantly higher (by approximately 3-fold) than in M202 at all time points, from day 0 to day 6 (Figure 1). The mRNA level of *Os07g47670* was induced both in M202 and M202(Sub1A) under submergence, which is not consistent with microarray results.

Sequence alignment and biochemical property of the Os07g47670 protein

The amino acid sequence of the Os07g47670 protein was used as a query sequence to search the databases using BLASTP. Ten homologous genes were identified. There are one homologous gene (Os02g37930.1) in rice, two (GRMZM2G159691, GRMZM2G010783) in maize, two (AT3G05550.1, AT5G27760.1) in *Arabidopsis*, two (Potri.005G024500.1, Potri.013G015400.1) in poplar, two (Sb04g024580.1, Sb02g042700.1) in Sorghum, and one (Bradi1g18030.1) in Brachypodium, respectively. The eleven proteins are highly homologous, and all belong to hypoxia-responsive family proteins with highly conserved amino acid sequences (Figures 2 and 3). The amino acid sequences, number of amino acids, theoretical molecular weight, theoretical pI recombinant protein solubility of Os07g47670 are shown as Supplementary Table 2. No disordered regions are found in the Os07g47670 protein (Supplementary Figure 1).

Prediction of the *Os07g47670* promoter

The main cis-regulatory elements that are characteristic of a promoter sequence are revealed by PlantCARE prediction, including a Py-rich stretch element (-988 to 998 bp), conferring high transcription levels. Importantly, a number of cis-regulatory elements related to abiotic stress were found in 5 promoter of the Nipponbare gene, such as two anaerobic response elements (ARE) involved in anaerobic induction, three MYB binding site elements (MBS) involved in drought-inducibility, and one TC-rich repeats related to defense and stress responsiveness. One of the ARE is present on the plus-strand (-1807 to

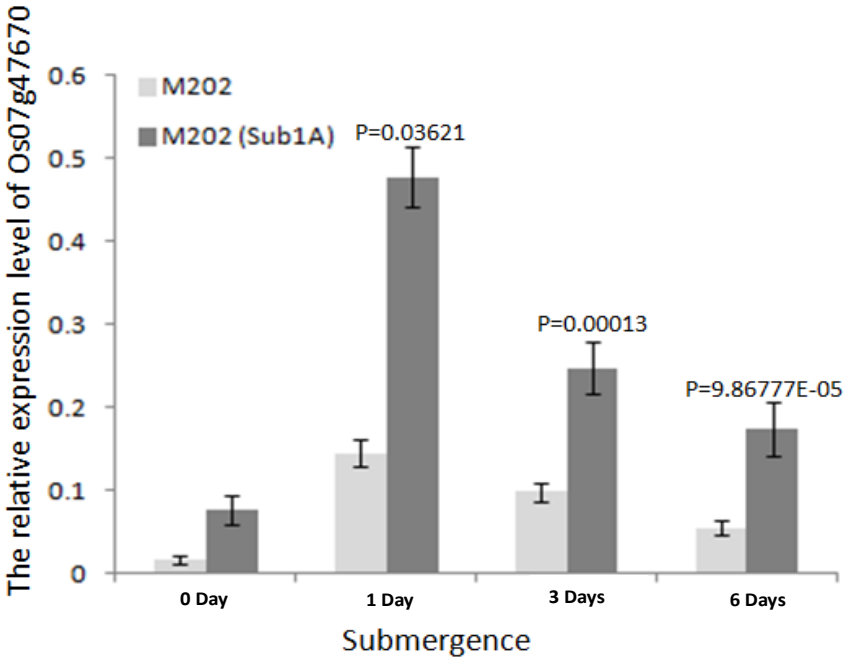

Figure 1. Expression level of the *Os07g47670* gene under submergence in M202 and M202(Sub1A). Fourteen-day old M202 and M202(Sub1A) seedlings were subject to submergence treatment for 0, 1, 3, and 6 days. The leaves were used to extract total RNA samples, which were used in qRT-PCR experiments. The expression levels of *Os07g47670* were calculated using 2^ddCt values. Each bar represents the mean±SD of 3 independent biological replicates.

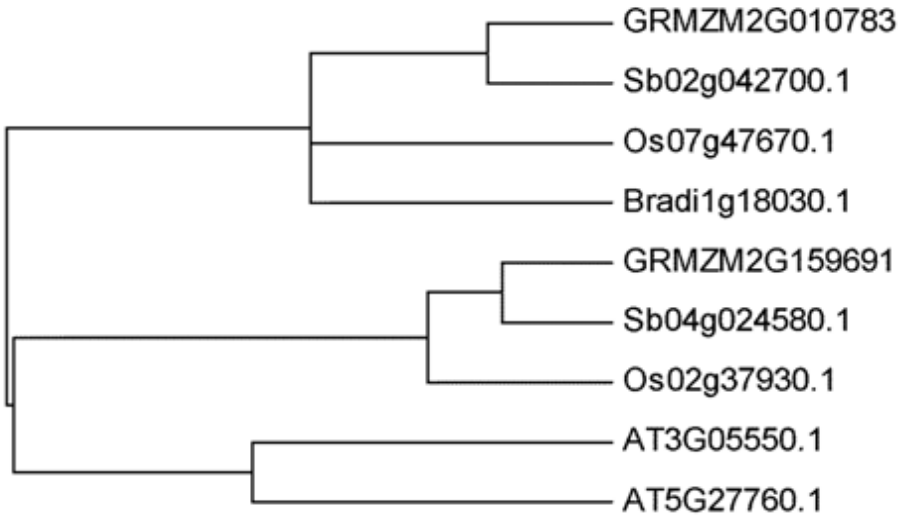

Figure 2. Phylogenetic tree of Os07g47670 and its homologous proteins of *Arabidopsis*, rice, maize, poplar, Sorghum, and Brachypodium. Full-length amino acid sequences were used in the multiple sequence alignment analysis using ClustalX. The phylogenetic tree of the aligned protein sequences was constructed using MEGA.

-1813 bp), and the other on negative-strand (-1845 to 1851 bp). One TC-rich repeats is present at -1389 to -1399 bp at negative strand. For the three MBS elements, two are present on the plus-strand (-1445 to -1451 and

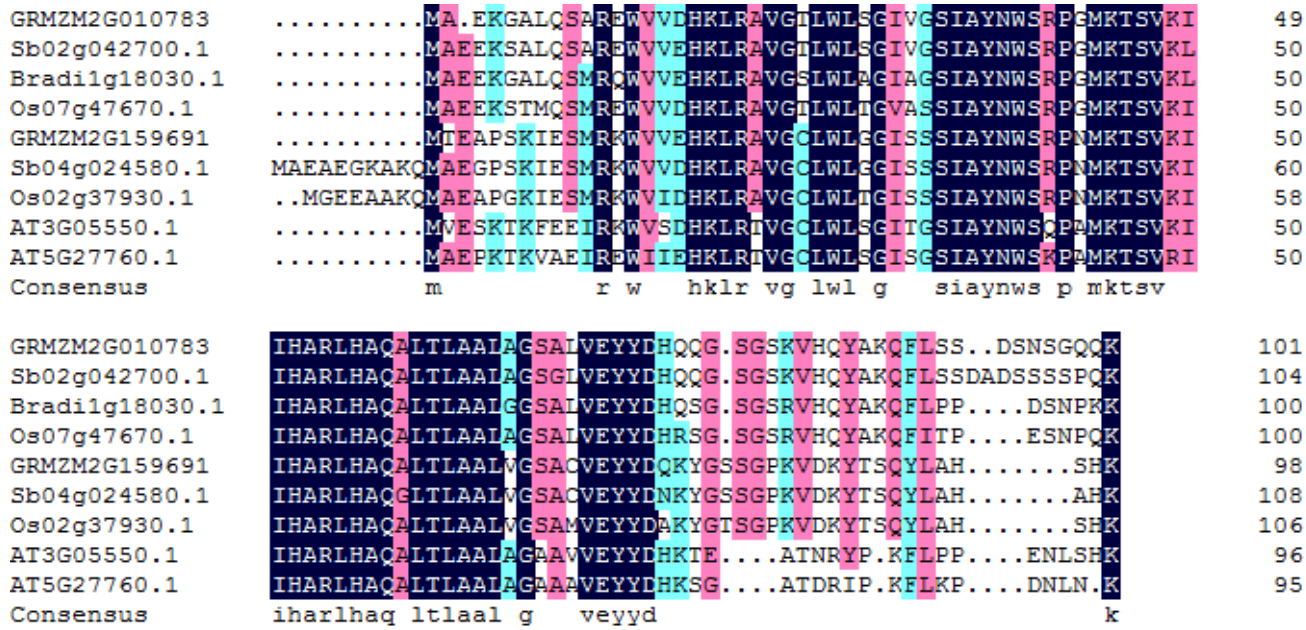

Figure 3. Sequence alignment of Os07g47670 and its homologous proteins. Multiple sequence alignment analysis was performed using ClustalX. Black color indicates amino acid residues conserved in every protein; dots represent gaps in amino acid sequences.

Table 2. Putative cis-acting elements in the *Os07g47670* gene promoter.

Site name	Position	Strand	Sequence	Function
5′UTR py-rich stretch	-988~ -998	+	TTTCTTCTCT	conferring high transcription level
ARE	-1807~-1813	+	TGGTTT	anaerobic induction
ARE	-1845~-1851	–	TGGTTT	anaerobic induction
TC-repeats	-1389~ -1399	–	ATTTTCTTCA	Cis-acting element involved in defense and stress responsiveness
MBS	-1630~-1636	+	TAACTG	MYB binding site involved in drought-inducibility responsiveness
MBS	-1445~ -1451	+	TAACTG	MYB binding site involved in drought-inducibility responsiveness
MBS	-1219~-1225	–	CGGTCA	MYB binding site
ABRE	-1249~ -1259	–	TACGTG	involved in the abscisic acid responsiveness
TCA-element	-1960~ -1969	+	CCATCTTTTT	involved in salicylic acid responsiveness
TCA-element	-997~ -1006	–	GAGAAGAATA	involved in salicylic acid responsiveness
AuxRR-core	-1186~ -1193	+		

-1630 to -1636 bp) and one on the negative strand (-1219 to -1225 bp). In addition, there are four cis-regulatory elements involved in hormone responsiveness, including one ABRE involved in abscisic acid responsiveness, one AuxRR-core involved in auxin responsiveness, and two TCA-elements related to salicylic acid responsiveness. The ABRE is at -1249 to -1259 bp on the negative-strand. For the two TCA-elements, one is located at -997 to -1006 bp on the negative strand and the other at -1960 to -1969 bp on the plus strand. The AuxRR-core is located at -1186 to -1193 bp on the plus strand (Table 2).

Expression of the *Os07g47670* gene in different tissues

The mRNA levels of the *Os07g47670* gene in different tissues were determined using qRT-PCR. At the vegetative stages of one and two-week old plants, the mRNA level of *Os07g47670* in shoots is low both in M202 and M202(Sub1A), but is high in roots. The transcript levels of *Os07g47670* in sheath, root, and leaf tissues in one-month old plants are higher than in shoots, but are lower than in roots, of one- or two-week old plants. At the

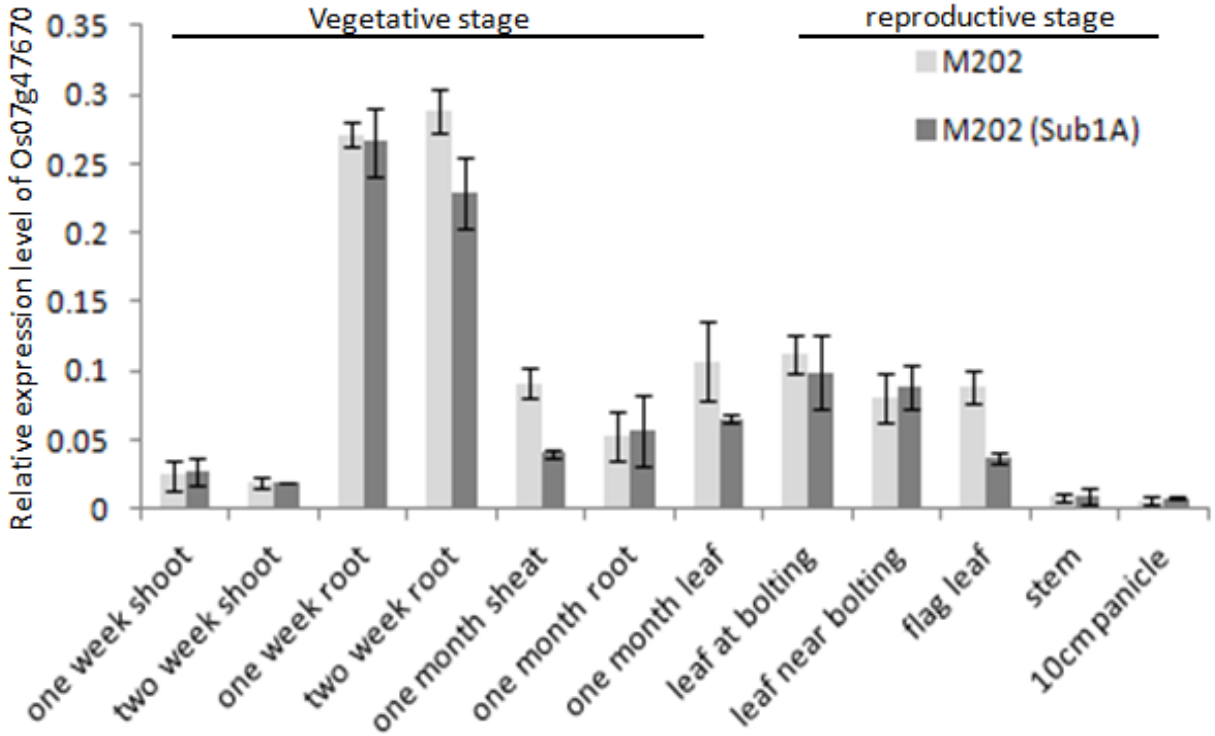

Figure 4. Transcript levels of the *Os07g47670* gene in various tissues at different stages. The expression levels of *Os07g47670* were calculated using 2^ddCt values. Each bar represents the mean±SD of 3 independent biological replicates.

reproductive stage, the expression levels of *Os07g47670* are high in leaves, including leaves at bolting, leaves near bolting, and the flag leaf, but are low in stems and panicles. The highest mRNA levels of *Os07g47670* are expressed in roots of one- or two-week old plants (Figure 4).

Os07g47670, ADH1, SLR1, SLRL1, and *Sub1A* are co-expressed under submergence

Sub1A is an important gene for submergence tolerance in rice and it confers submergence tolerance by augmenting accumulation of the GA signaling repressors SLR1 and SLRL1. *ADH1* is a well-known marker gene for submergence tolerance. Therefore, we carried out real-time RT-PCR to assess possible co-expression of these genes and *Os07g47670* under submergence. Under submergence, the expression levels of *Os07g47670, ADH1, Sub1A, SLR1*, and *SLRL1* were all induced, reaching the peak at day 1 of submergence, and then gradually decreased from day 3 to day 6 under submergence. The *Os07g47670* gene exhibits a similar expression pattern with the *Sub1A, ADH1, SLR1*, and *SLRL1* genes during submergence. Therefore, the *Os07g47670* gene is co-expressed with *Sub1A*, *ADH1*, *SLR1*, and *SLRL1* during submergence stress (Figure 5).

Os07g47670 and *Sub1A* are co-regulated by GA and ACC

Sub1A encodes an ethylene-responsive transcription factor (ERF) and is strongly induced by ACC, the precursor of ethylene. *Sub1A* confers submergence tolerance by modulating the GA signal pathway in rice. Therefore, we performed real-time PCR to assess the expression of *Os07g47670* and *Sub1A* under GA and ACC treatments, respectively. With ACC treatment, *Os07g47670* and *Sub1A* gene transcript levels in M202(Sub1A) were strongly induced. The mRNA level of *Os07g47670* in M202(Sub1A) was induced to about 3.0 fold compared to mock (without ACC treatment), indicating that ACC stimulates the expression of *Os07g47670* (Figure 6a). However, the mRNA level of *Os07g47670* in M202 did not significantly increase with ACC treatment (Figure 6b). With GA treatment, the mRNA levels of *Os07g47670* and *Sub1A* were modestly decreased in M202(Sub1A), indicating that GA suppresses the expression of Os07g47670 and *Sub1A*, in contrast to ACC action. These results demonstrate that *Os07g47670* and *Sub1A* share the same expression patterns in

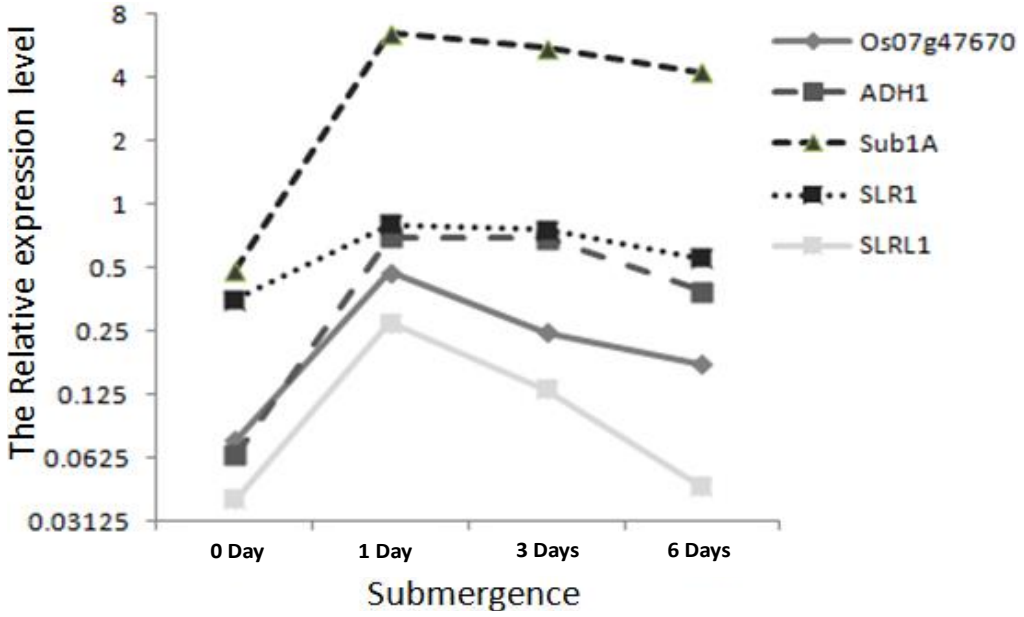

Figure 5. Time course of *Os07g47670*, *SLR1*, *SLRL1*, *ADH1*, and *Sub1A* expression under submergence. Fourteen-day old seedlings were subjected to submergence and GA treatments. The mRNA levels in leaves were determined by qRT-PCR. The expression levels were calculated using 2^ddCt values. Each bar represents the mean±SD of 3 independent biological replicates.

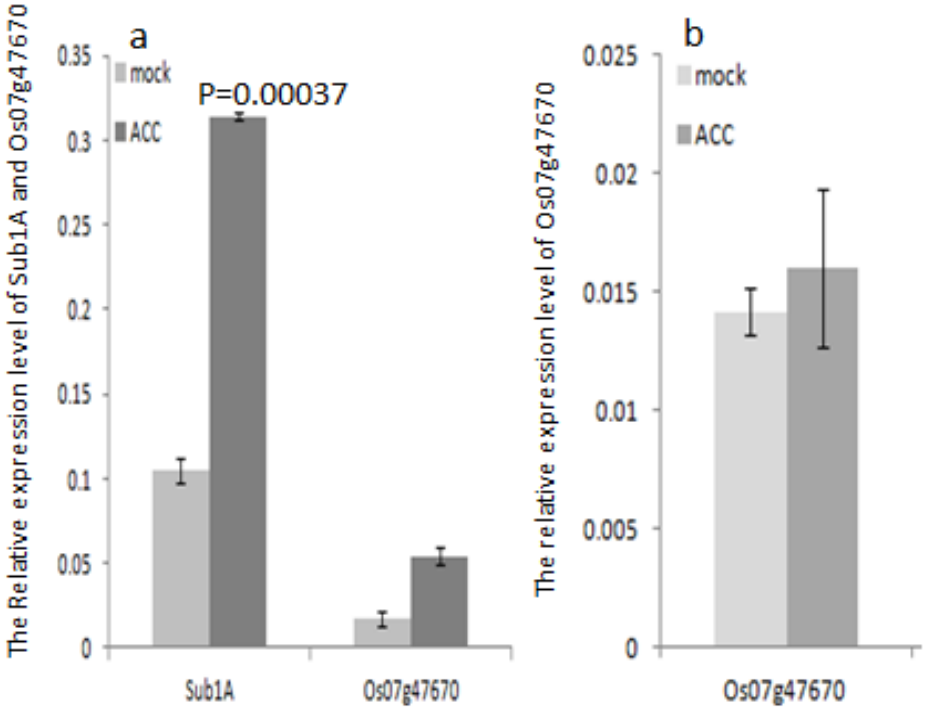

Figure 6. Expression levels of *Os07g47670* and *Sub1A* after ACC treatment. **a.** Expression levels of *Os07g47670* and *Sub1A* in M202(Sub1A) with and without ACC treatment. **b.** Expression levels of *Os07g47670* in M202 with and without ACC treatment. 14-day old M202 and M202(Sub1A) seedlings were subject to mock and ACC treatment, respectively. The transcript levels of *Sub1A* and *Os07g47670* were determined by qRT-PCR. The expression level was calculated using its 2^ddCt value. Each bar represents the mean±SD of 3 independent biological replicates.

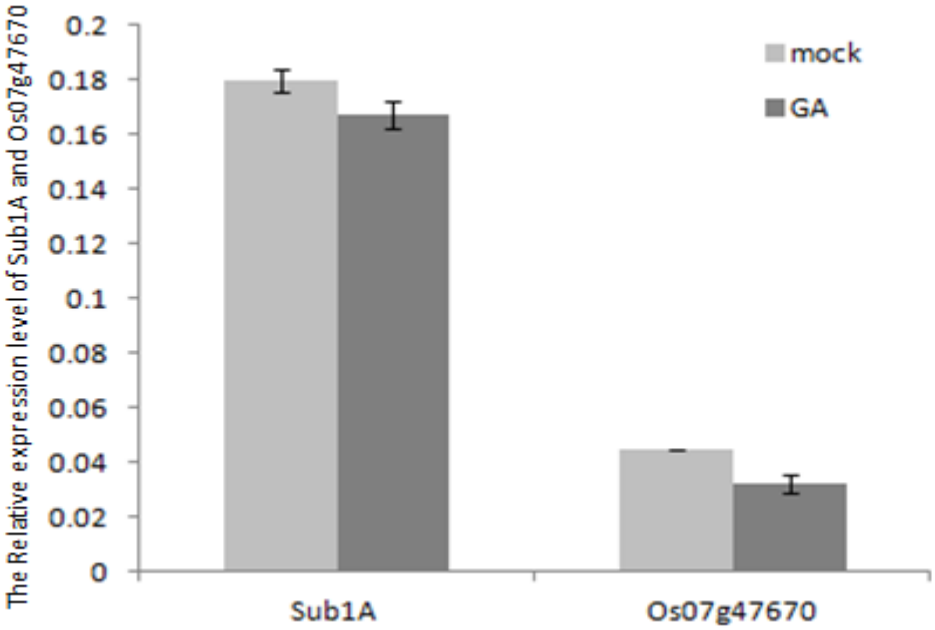

Figure 7. Expression level of *Os07g47670* in M202(Sub1A) after GA treatment. 14-day old M202(Sub1A) seedling were subject to mock and GA treatment, respectively. The expression level of *Os07g47670* was measured by real-time PCR and calculated using its 2^ddCt value. Each bar represents the mean±SD of 3 independent biological replicates.

response to GA and ACC treatments (Figure 7).

DISCUSSION

The candidate gene approach has been at the forefront in studying many biological aspects. The use of bioinformatics tools is an affordable, fast, and efficient method for researchers to mine candidate genes responsive to various environmental stress or disease challenges. Submergence is one of the major constraints to rice production. In the present study, we attempted to identify novel genes associated with submergence tolerance in a silico approach. The microarray data were downloaded from the NCBI GEO database and analyzed for differential gene expression patterns between M202 and M202(Sub1A) under submergence stress. One novel gene, *Os07g47670*, was found to respond to submergence. The mRNA levels of the *Os07g47670* gene are low in M202 both in normal and submergence conditions, while are strongly induced in M202(Sub1A) during submergence. BLASTP and ClustalX analyses show that the Os07g47670 protein is highly homologous to protein members that belong to a hypoxia-responsive protein family. Analysis of the *Os07g47670* promoter reveals that there are two ARE cis-regulatory elements associated with hypoxia responsiveness. This *in silico* prediction was based on best available knowledge on cis-elements. Therefore, the prediction indicates that *Os07g47670* is a novel gene responsive to submergence stress in rice.

In the submergence-intolerance cultivar M202, the Sub1 region, covering 182 kb on chromosome 9, encodes two ERF genes, *Sub1B* and *Sub1C*. In tolerance near-isogenic line, M202(Sub1A), this locus encodes an additional ERF gene, namely *Sub1A*. Thus, *Sub1A* mediates the extinguished submergence tolerance of other cultivated rice. Although the molecular mechanism of *Sub1A* has been reported (Fukao and Bailey-Serres., 2008), genes that interact with Sub1A to confer submergence tolerance remain unclear. In the present study, in the absence of the *Sub1A* gene, the transcript levels of *Os07g47670* remain low in M202 both in normal and submergence conditions. On the contrast, in the presence of the *Sub1A* gene, the *Os07g47670* mRNA level is significantly increased in M202(Sub1A) during submergence. Thus, the induction of *Os07g47670* expression is specific to *Sub1A*.

Sub1A is strongly induced by ACC treatment (Fukao and Bailey-Serres., 2008). In M202 absent of Sub1A, the *Os07g47670* transcript level was not significantly increased with ACC treatment. However, in the presence of Sub1A, the expression level of *Os07g47670* in M202(sub1A) is significantly induced by ACC treatment. *ADH1, SLR1*, and *SLRL1* are submergence tolerance marker genes (Fukao and Bailey-Serres, 2008). *Os07g47670* responds to submergence stress similarly

as *ADH1, SLR1, SLRL1*, and *Sub1A*. Thus, *Os07g47670* is co-expressed with *Sub1A, ADH1, SLR1*, and *SLRL1* under submergence, indicating that they may function in the same pathway. Our results confirm that *Os07g47670* is a novel gene responsive to submergence stress. Therefore, the silico approach to identify candidate gene of submergence tolerance has demonstrated its validity.

Our results suggest that Sub1A regulates the expression of *Os07g47670*. Sub1A is an ERF transcription factor and can potentially bind to the two identified ARE cis-elements in the *Os07g47670* promoter to activate the promoter. Alternatively, *Sub1A* can activate other transcription factors that bind to the two ARE cis-elements and activate *Os07g47670* expression. It remains to be determined how *Sub1A* regulates *Os07g47670* expression.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENTS

We thank Pro. Xuewei Chen (Sichuan Agricultural University) for providing M202 and M202(Sub1A) seeds. We are grateful to Dr. Chern Mawsheng (University of California, Davis) for his critical reading and editing of the manuscript. This work was supported by National Natural Science Foundation (31271741) and the Hubei Province Natural Science Foundation (2011CDB006 and 2012FFA051).

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Zhuang J, Cai B, Peng RH, Zhu B, Jin XF, Xue Y, Gao F, Fu XY, Tian YS, Zhao W, Qiao YS, Zhang Z, Xiong AS, Yao QH (2008). Genome-wide analysis of the AP2/ERF gene family in *Populus trichocarpa*. Biochem. Biophys. Res. Commun. 371:468-474.

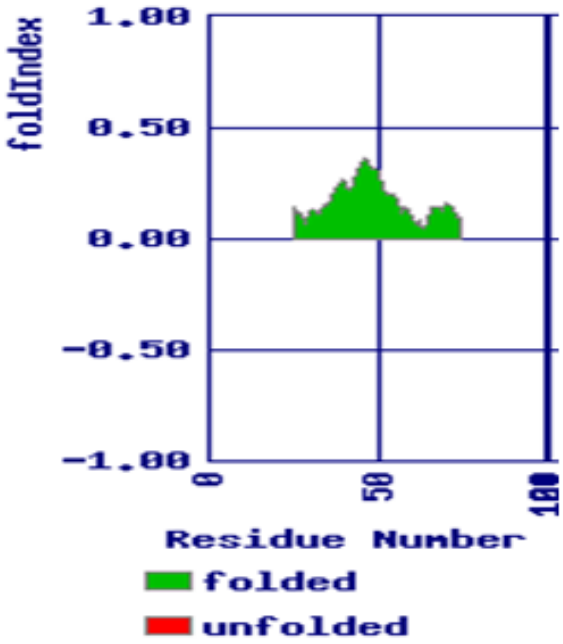
Supplementary Table 1. Microarray data of the Os07g47670 gene under submergence.

log_2 s/m_0d	log_2 s/m_1d	log_2 s/m_6d	log_2 1d/0d_sub1	log_2 6d/0d_sub1	log_2 1d/0d_m202	log_2 6d/0d_m202
-2.66329	2.254901	0.706373	2.743458	1.576116	-2.17473	-1.79355

s and sub1, M202(Sub1A); m, M202; 0d, 1d, 6d, number of days subject to submergence.

Supplementary Table 2. The composition and physical and chemical characterization of amino acid sequences in Os07g47670.

Genbank No.	Number of amino acid	Theoretical Mw/Da	Theoretical pI	Percentage of Aliphatic amino acid /%	Percentage of Aromatics amino acid /%	Percentage of Positive amino acid /%	Percentage of Negative amino acid/%	Recombinant Protein Solubility(per cent of insolubility)/ %
Os07g47670	101	11269.85	9.63	84	8	17	8	97.4



Supplement Figure 1. Prediction of the folding state of Os07g47670.

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Full Length Research Paper

Construction of a full-length cDNA library and analysis of expressed sequence tags in white jute (*Corchorus capsularis* L.)

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White jute (*Corchorus capsularis* L.) is recognized as an important industrial raw material fibre crop owing to its elite characters. However, little information is known about its molecular basis and genomics. In this study, a complementary DNA library of white jute was constructed and expressed sequence tags (ESTs) were characterized. The titers of original and amplified libraries were 2.32×10^7 and 1.07×10^9 pfu/mL, respectively. The recombinant frequency was 98.3% in the library. Most of the sequences ranged from 500 to 1500 bp with an average length of 750 bp. Results show 203 (73%) ESTs exhibited significant similarity with known or putative functional nucleotide sequences in the GenBank databases. Cluster analysis allowed the identification of 61 unique sequences. These genes were classified into six types by Gene Ontology (GO) annotation. The results also indicated that unigenes of *C. capsularis* have higher homology to *Populus trichocarpa*, *Ricinus communis* and *Corchorus olitorius*. This report will provide a valuable resource for the further investigations in the gene cloning, transcription or expression for white jute.

Key words: White jute, *Corchorus capsularis*, cDNA library, construction, ESTs, analysis.

INTRODUCTION

Jute (*Corchorus capsularis* L.) is an important bast fibre crop extensively grown in Southeast Asian countries. Jute fibres exhibit a characteristically high luster, good moisture absorption performance, rapid water loss capacity and easy degradation (Zhang et al., 2013). The textile and paper industry are interested in its potential as

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Abbreviations: ESTs, Expressed sequence tags; **GO,** gene ontology; **cDNA,** complementary DNA; **SMART,** switching mechanism at 5' end of the RNA transcript; mRNA, messenger RNA; **SSR,** simple sequence repeat; **BGI,** Beijing genomics institution; **NCBI,** national center for biotechnology; **PVPP,** polyvinylpyrrolidone.

an important ingredient for producing paper, fine textiles as well as a renewable source for biofuel (Wazni et al., 2007). In recent years, with increasing uses of jute in diversified industries, there is a growing demand for high-yielding new cultivars. This has posed a serious challenge because no new breeding approaches have been developed for jute over the past seven decades (Kundu et al., 2015). Instead of using traditional breeding methods, jute breeders and biologists are now turning their attention to use molecular tools to improve agronomic traits (Khatun, 2007). However, many potentially fruitful research avenues, especially large-scale gene expression surveys and development of molecular genetic markers have been limited by a lack of sequence information in public databases (Zhang et al., 2011a). For a non-model species such as jute, with no prior genomic knowledge and resources (only 2,036 nucleotide and 54 EST sequences in NCBI database) (Kundu et al., 2015), it is critical to isolate, identify and understand the expressed sequences, with a long term goal of genetically modifying jute.

Generation and characterization of gene libraries is the priority for plant breeders because it is a fundamental platform in diverse aspects as genetic and physical mapping, molecular marker, new genes isolation and identification, and comparative genomics research (Talon and Gmitter, 2008; Zhang et al., 2011b; Zheng et al., 2011; Zhang et al, 2014). Expressed sequence tag (EST) projects provide a very useful and quick means of accessing gene sequence and expression information (Manickavelu et al., 2012). Some reports have proven that projects based on ESTs are powerful tools for both the analysis of gene expression patterns in a given tissue or at specific developmental stages (Chen et al., 2012; Tran et al., 2011; Wang et al., 2011b; Yang et al., 2009b; Zhang et al., 2011a). Moreover, ESTs from complementary DNA (cDNA) clones are inexpensive and efficient gene discovery tools (Wang et al., 2011a; Xiao et al., 2011; Yamagishi et al., 2011). As a molecular basis of information on whole genomes, the accumulation of ESTs is a promising strategy for studies in plant molecular biology (Rudd, 2003). These technologies are particularly important for plants lacking genomic sequence information such as *C. capsularis*.

Significant progress has been made in the last decade to understand the genome sequences of *Corchorus* species by ESTs analysis. So far, partial cDNA sequences of putative phosphate transport ATP-binding protein gene of C. *capsularis* var. CVL-1 were submitted (Islam et al., 2005). Amherst published partial cDNA base sequences of NADH dehydrogenase (ndhF) gene of C. *capsularis* (Whitlock et al., 2003). Determined complete cDNA sequence of caffeoyl-CoA-O-methyltransferase and cinnamyl alcohol dehydrogenase which are two of the three genes involved in lignin biosynthesis of *capsularis*. Basu et al., 2003a; Basu et al., 2003b Samanta et al. (2015) reported that WRKY transcript factor was a most important transcript in fibre development process of jute by EST analysis. In fact, there are some other major challenges for jute except poor fibre quality, which include susceptibility of the jute crop to fungal diseases, photoperiod sensitivity, and low yield under unfavorable growth conditions. The long term goal of this project is to better understand the jute genome and to produce transgenic jute varieties that will have higher fibre yield and quality than that of current cultivars without compromising their other important agronomic traits such as disease resistance, photoperiod insensitivity, strong and lustrous fibres, etc.

In this study, we aimed to construct a full-length cDNA library, to conduct EST analyses, and to analyze and classify gene functions from the leaves of '179' to lay foundations for the further utilization of the gene resources from '179' for the improvement of white jute by genetic transformation.

MATERIALS AND METHODS

Plant materials

The plant material used in this study was *C. capsularis* cultivar "179". "179" which has a fibre yield about 10% higher than that of Yue-yuan No. 5 (used as check) and also a high resistance to anthracnose, which is a potential material for molecular and biology study (Lu et al., 1983). The seedlings of "179" were grown in a greenhouse under natural conditions, then transplanted and grown under normal field management in Fujian Agriculture and Forestry University. Tender leaves were separated from the plants and immediately frozen in liquid nitrogen, then stored at -80°C until use.

Construction of the normalized cDNA library

Total RNA was extracted according to the protocol of RP3301 RNA extraction kit (Bioteke Corporation) with modified. Messenger RNA (mRNA) was isolated from total RNA using Oligotex (Qiagen, The Netherlands) mRNA extraction kit. The total RNA and mRNA quantities were determined spectrophotometrically at wavelengths of 230, 260, and 280 nm. The integrity of the total RNA and mRNA was verified by running samples on 1.1% agarose gels.

cDNA library construction and characterization

First and double-stranded cDNAs were synthesized as described in the manual of the SMART cDNA library construction kit (Clontech, USA). 50% of the double-stranded cDNAs were digested with proteinase K and fractionated by Creator^{TM} SMART^{TM} cDNA Library Construction Kit. The cDNA fragments were selected and purified and cloned into a pMD-18T vector; the recombinant DNA was put in *vitro* packaged using competent cell and LB liquid medium at 37°C for 1 h. *Escherichia coli* DH5α were infected with the phage from cDNA library to determine the titer of original and amplified library. The percentage of recombinant clones was determined by screening for blue/white plaques on medium containing X-gal and isopropyl β-D-1- thiogalactopyranoside. Finally, the titer and the recombination frequency of the library were calculated by the number of blue and white plaques: library titer P (pfu/mL) = number of plaques × dilution factor × 10^3 / volume of phage plates (μL), and recombination frequency (%) = number of white plaques / total

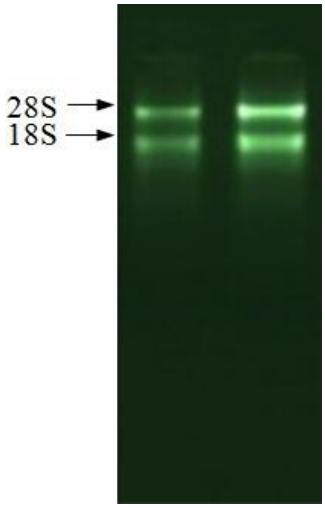

Figure 1. 1.1% agarose gel electrophoresis of total RNA from tender leaves of white jute cultivar '179'.

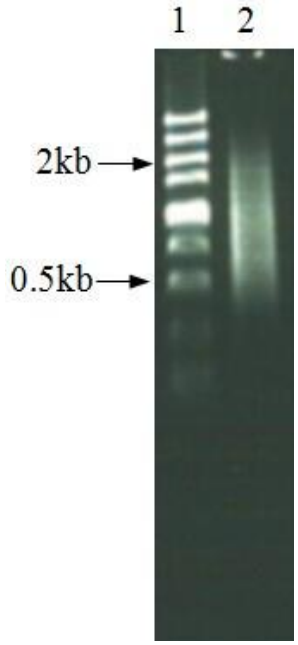

Figure 2. 1.1% agarose gel electrophoresis of double-strand cDNA. **1** represent DL5000 Marker; **2** represent the double-strand cDNA.

number of plaques. According to the sequences of two ends of the λ phage vector, forward primer was designed as 5'- CGCCA GGGTT TTCCC AGTCA CGAC-3', and the corresponding reverse primer was designed as 5'- GAGCG GATAA CAATT TCACA CAGG -3'. PCR procedures were carried out as follows: initial denaturation at 94°C for 5 min; 94°C for 30 s, 46°C for 45 s and 72°C for 2 min (35 recycles in total), and a primer extension at 72°C for 7 min. After amplification by plasmid PCR and identification by positive clone screening, suspected strains were combined with the same amount of 30% glycerin and then sent to Beijing Genomics institution (BGI, China) for sequencing.

Homology comparisons and analysis of ESTs

ESTs obtained were evaluated using software DNAstar and Chromas, and were spliced by software Phrad. Each edited EST was translated in reading frames and compared with the non-redundant database at the National Center for Biotechnology (NCBI) using the BLASTX program, which compares translated nucleotide sequences with protein sequences. ESTs longer than 100 bp and containing no more than 4% ambiguity were considered useful for data analysis. Using the BLAST service, at NCBI (http:// www.ncbi.nlm.nih.gov), sequences were searched against the protein and nucleic acid databases. Sequence similarities identified by the BLAST programs were considered to be statistically significant at an E-value of $\leq 10^{5}$. Molecular function of annotated genes was classified by Gene Ontology.

RESULTS

Construction of full-length cDNA library

Isolation of high quality total RNA is a critical step for constructing a cDNA library. In this study, the total RNA electrophoresis on 1.1% agarose gels showed distinct 18S and 28S (Figure 1), indicating good quality of total RNA. The optical density ratios at A260/280 and A260/230 were 1.99 and 2.26, respectively, suggesting little contamination of polysaccharides and proteins. The density of isolated total RNA was 418 ng/μL. The titer of the original library was approximately 2.32×10^{7} pfu/mL, much higher than 1.0×10^{6} pfu/mL, the estimated criteria for an available cDNA library. The amplified library was up to 1.07×10^{9} pfu/mL. The percentage of recombinants calculated through blue/white plaques was 98.3%. Size fractionated double-strand cDNA was visualized as a smear on the agarose gel (1.1%) with a size ranging from 0.6 to 1.5 kb (Figure 2). Further confirmation was obtained with electrophoresis PCR products of 44 randomly selected clones. The size of the cDNAs ranged from 0.5 to 1.5 kb, with an average size of 0.75 kb (Figure 3). Based on the above observation, the constructed cDNA library reached the criteria (library content and cDNA integrity) for isolating full-length expressed genes in *C. capsularis*.

Obtaining and splicing effective EST sequences

The sequences were trimmed off by their vector, adaptor, poly(A) tail, and low-quality sequences and filtered for minimum length (100 bp), resulting in a total of 219 high quality ESTs (Table 1), which percentage was 77.42%. The length of all the ESTs was from 106 to 1386 bp with an average size of 531 bp. Using Phrap software to

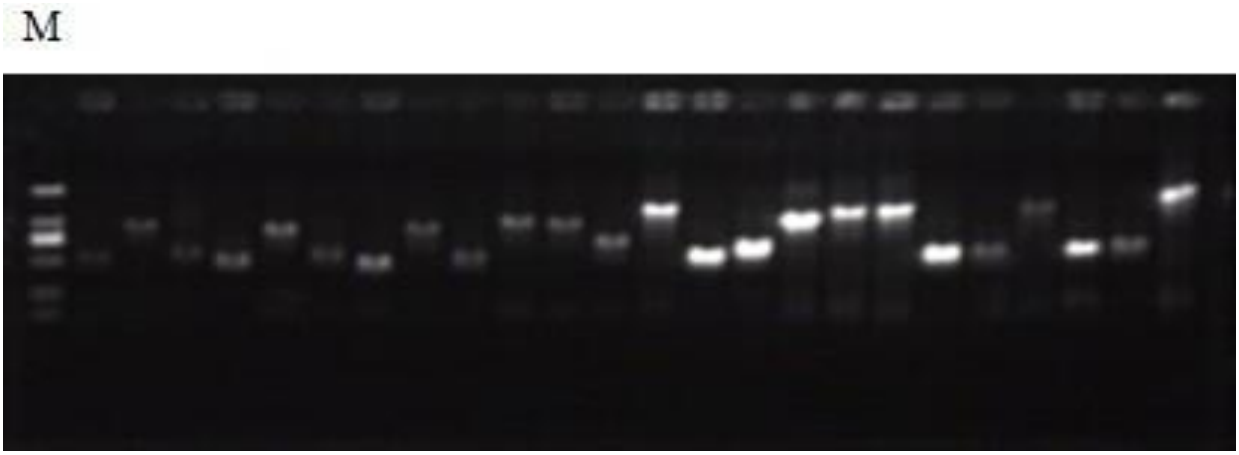

Figure 3. Twenty-four (24) clones in the library were selected randomly to evaluate their insert sizes. The size of the cDNAs ranged from 0.5 to 1.5 kb, with an average size of 0.75 kb. M represents DL2000 Marker.

Table 1. cDNA library, ESTs and cluster statistics of white jute cultivar '179'.

cDNA Library characteristics	Eigen value
Titer of cDNA library (pfu mL^{-1})	2.32×10^{7}
Recombination rate	98.3%
Average cDNA insert size	0.75 kb
Sequences passing quality check	219 (77.42%)
Average length	531bp
Singletons	37
Contigs	24

conduct cluster-analysis and post-splicing, a total of 61 non-repetitive sequences (unigenes) were found including 24 contigs and 37 singleton ESTs.

Functional analysis and classification based on gene ontology

To provide a deeper understanding of the gene expression in jute, 41 unigenes including 203 ESTs bearing known functions or assumed functions based on BLASTX were analyzed (Table 2). The results reveal putative functions of 41 unigenes include cold-inducible and light-inducible protein, chloroplast precursor, 50S ribosomal protein, oxygen-evolving enhancer protein and other hypothetical protein. There were other six unigenes which could be matched and annotated but which function could not be known. Moreover, a total number of 14 unigenes could not be matched and annotated, indicating represent genes are likely new genes with new function. GO has been used widely to predict gene functions and classification (Ashburner et al., 2000; Wang et al., 2007). In our study, there were 19 genes be classified successfully according to their function, which were clustered into 6 functional categories involved in the cellular processes of energy production and conversion, metabolism, translation, ribosomal structure and biogenesis (Figure 4). It revealed that the encoded energy production and conversion-related genes present the largest number of ESTs, with a total of 7 occupying 36.84% of the functionally described genes. Carbohydrate transport and metabolism-related genes also present a larger number of ESTs, with a total of 6 occupying 31.58% of the functionally described genes. Amino acid transport and metabolism genes occupied 10.53% of all described genes, with the same percentage of translation, ribosomal structure and biogenesis genes. Two types of genes had the least percentage of 5.26%, which were inorganic ion transport and metabolism genes and lipid transport and metabolism genes.

Homologous analysis

Sixty (61) unigenes were analyzed by BlastX and DNAman software, among which 47 unigenes were homologous with

Table 2. Annotations and functional classification of unigenes.

Putative function	Species matched	Matches no	E-value
Repeat-containing protein	*Glycine max*	ref|XP_003519707.1|	2.00E-61
Predicted protein	*Populus trichocarpa*	ref|XP_002312449.1|	2.00E-62
Predicted protein	*Populus trichocarpa*	ref|XP_002308765.1|	1.00E-57
Putative cold-inducible protein	*Camellia sinensis*	gb|ACB20694.1|	8.00E-43
Predicted protein	*Populus trichocarpa*	ref|XP_002303722.1|	2.00E-47
50S ribosomal protein L34, chloroplast precursor, putative	*Ricinus communis*	ref|XP_002522558.1|	2.30E-30
Ycf3	*Ranunculus macranthus*	gb|AAZ03964.1|	2.00E-16
Chalcone isomerase	*Gossypium hirsutum*	gb|ADG27840.1|	1.00E-88
Similar to Os01g0104000	*Vitis vinifera*	ref|XP_002263812.1|	4.00E-58
Ascorbate peroxidase	*Zea mays*	ref|NP_001170482.1|	6.00E-07
Oxygen-evolving enhancer protein3,chloroplast precursor, putative	*Ricinus communis*	ref|XP_002518571.1|	3.00E-25
Hypothetical protein	*Vitis vinifera*	ref|XP_002273533.1|	3.00E-31
Conserved hypothetical hypothetical protein	*Ricinus communis*	ref|XP_002517183.1|	5.00E-64
Light-inducible protein ATLS1	*Elaeis guineensis*	gb|ACF06473.1|	4.00E-54
Hypothetical protein osi_08842	*Oryza sativa* Indica Group	gb|EEC73954.1|	4.00E-28
Hypothetical protein	*Vitis vinifera*	ref|XP_002284937.1|	9.00E-46
Cytoplasmic ribosomal protein S13	*Solanum lycopersicum*	ref|NP_001234162.1|	9.00E-59
Hypothetical protein VITISV_001773	*Vitis vinifera*	emb|CAN64991.1|	6.00E-50
Predicted protein	*Populus trichocarpa*	ref|XP_002313119.1|	4.00E-37
Predicted protein	*Populus trichocarpa*	ref|XP_002306249.1|	4.00E-10
Hypothetical protein	*Vitis vinifera*	ref|XP_002263049.1|	7.00E-83
Serine hydroxymethyl transferase	*Corchorus olitorius*	gb|ABS72195.1|	8.00E-20
NADH dehydrogenase subunit 4	*Nicotiana tabacum*	dbj|BAD83480.2|	4.00E-10
Ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit 1A	*Corchorus olitorius*	gb|ABS72189.1|	3.00E-24
Tonoplast intrinsic protein putative	*Ricinus communis*	ref|XP_002531978.1|	1.00E-123
Unknown protein	*Nicotiana tabacum*	gb|AAA84679.1|	3.00E-06
Predicted protein	*Populus trichocarpa*	ref|XP_002327750.1|	1.00E-168
Conserved hypothetical protein	*Ricinus communis*	ref|XP_002525607.1|	4.00E-16
Acyl-coa-binding protein	*Jatropha curcas*	gb|ADB85092.1|	2.00E-40
Hypothetical protein osi_13874	*Oryza sativa* Indica Group	gb|EAY92161.1|	2.00E-22
Fructose -1, 6-bisphosphate aldolase class 1	*Carica papaya*	gb|AER26531.1|	2.00E-45
Ribulose-1, 5-bisphosphate carboxylase/oxygenase Small subunit 1a	*Corchorus olitorius*	gb|ABS72189.1|	1.00E-87
Hypothetical protein MTR_5g050970	*Medicago truncatula*	ref|XP_003614382.1|	2.00E-92
Predicted protein	*Populus trichocarpa*	ref|XP_002306693.1|	8.00E-88
Ribulose-1, 5-bisphosphate carboxylase/oxygenase Small subunit 1a	*Corchorus olitorius*	gb|ABS72189.1|	6.00E-76
Chloroplast precursor putative	*Ricinus communis*	ref|XP_002510603.1|	4.00E-60
Uncharacterized protein LOC100499745	*Glycine max*	ref|NP_001235442.1|	7.00E-54
Predicted protein	*Populus trichocarpa*	ref|XP_002316235.1|	8.00E-88
Hypothetical protein	*Vitis vinifera*	ref|XP_002263786.1|	1.00E-108
Ribulose-phosphate 3-epimerase, chloroplastic-like	*Glycine max*	ref|XP_003524706.1|	1.00E-114
Ribulose-1, 5-bisphosphate carboxylase/oxygenase Small subunit 1a	*Corchorus olitorius*	gb|ABS72189.1|	6.00E-90
Pantothenate kinase putative	*Ricinus communis*	ref|XP_002514412.1|	1.00E-139
3-oxo-5-alpha-steroid 4-dehydrogenase family protein	*Arabidopsis thaliana*	ref|NP_197105.1|	1.00E-23
Ribulose-1, 5-bisphosphate carboxylase/oxygenase Small subunit 1a	*Corchorus olitorius*	gb|ABS72189.1|	4.00E-92
NADH dehydrogenase subunit K	*Eucalyptus globulus* subsp. globulus	ref|YP_636303.1|	1.00E-140
Orf174	*Beta vulgaris* subsp vulgaris	dbj|BAD66815.1|	1.00E-08
Hypothetical protein POPTRDRAFT_712066	*Populus trichocarpa*	ref|XP_002304206.1|	7.00E-44

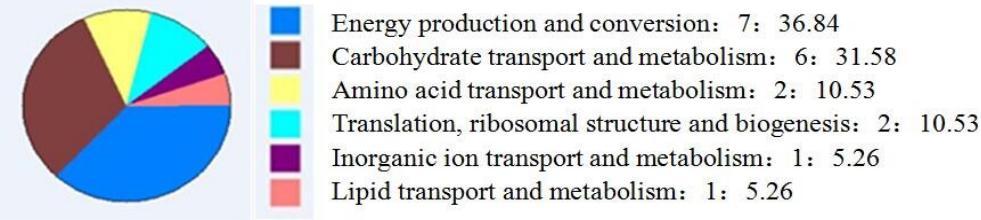

Figure 4. The GO classification of genes with function or putative function annotation.

Table 3. Numbers of homologous unigenes and matched species.

Species name	Homologous unigenes number	Percentage (%)
Populus trichocarpa	9	19.14
Ricinus communis	7	14.89
Corchorus olitorius	6	12.77
Vitis vinifera	6	12.77
Glycine max	3	6.38
Nicotiana tabacum	2	4.26
Oryza sativa Indica Group	2	4.26
Camellia sinensis	1	2.13
Arabidopsis thaliana	1	2.13
Ranunculus macranthus	1	2.13
Gossypium hirsutum	1	2.13
Zea mays	1	2.13
Elaeis guineensis	1	2.13
Solanum lycopersicum	1	2.13
Jatropha curcas	1	2.13
Carica papaya	1	2.13
Medicago truncatula	1	2.13
Beta vulgaris subsp. *vulgaris*	1	2.13
Eucalyptus globulus subsp. *globulus*	1	2.13

19 species (Table 3). The results show that unigenes of *C. capsularis* have highest homology to *Populus trichocarpa*, with a total of 9 occupying 19.14% of the analyzed unigenes. *Capsularis* unigenes also have higher homology to *Ricinus communis*, with a percentage 14.89% of the analyzed unigenes. The percentage of homologous unigenes to *Corchorus olitorius* of analyzed unigenes is 12.77%, with the same percentage of *Vitis vinifera.* Moreover, the *capsularis* unigenes are also homologous with other species, such as *Glycine max* (6.38%), *Nicotiana tabacum* (4.26%), *Oryza sativa* (Indica Group, 4.26%). While, the *capsularis* unigenes have lower homology percentage to *Camellia sinensis*, *Arabidopsis thaliana* and other 12 species, the percentage of analyzed unigenes is only 2.13%.

DISCUSSION

Quality of mRNA plays an important role in the construction of a full-length cDNA library, and high-quality mRNA is critical to the creation of full-length cDNA (Chen et al., 2012). Jute is a fairly primitive plant species somewhat different from those of other plant species (Samanta et al., 2011). It is difficult to isolate RNA from jute tissues because of the nature that much mucilage and phenolics are present in jute. The important steps taken into consideration in this study were the application of CsCl isopycnic centrifugation to remove insoluble poly-saccharides, and using polyvinylpyrrolidone (PVPP) to prevent oxidation of phenolics, as reported by Samanta et al (Samanta et al., 2011). The ratios of the optical density A260/230 and A260/280 were suggesting little contamination of polysaccharides and phenolics in isolated RNA, which was found to be appropriate for further downstream applications. A successful establish-ment of a cDNA library should contain almost all the expressed information possibly and should be examined with some quality index, such as abundance, integrity, and capacity (Zhang et al., 2012). Moreover, the titer of a cDNA library could be used as an evaluation criterion of the representativeness of the library (Yang et al., 2009a).

In general, it has been suggested that the titer of cDNA library should be above 1 × 10^6 pfu/mL. In the present study, the titer of the primary cDNA library was 2.32×10^7 pfu/mL, and the amplified library was up to 1.07×10^9 pfu/mL with a recombinant frequency of 98.3%, and the average insert size was 500 to 1500 bp. At the same time, the number of ESTs matching a particular gene should reflect the abundance of their corresponding cDNAs in the non-normalized library (Ewing et al., 1999). In our study, a total of 279 EST sequences were obtained after excluding the incomplete sequences, which provides the first nucleotide sequence data for white jute cultivar '179'. By functional speculation of the sequences of randomly chosen clones, 73% ESTs were supposed to be known putative function or have significant matches with hypothetical proteins, putative proteins, and 9% ESTs are assumed to be unknown proteins, 18% ESTs had no significant similarity to sequences in the public databases. Samanta et al. 2015 found 81% of the ESTs resulted by *C. capsularis* were similar with genes of known function, 2% showed homology with the putative sequences and 17% were similar to genes of unknown function (Samanta et al., 2015). The results indicated that the construction of the cDNA library of white jute was successful, which could serve as an important resource for the isolation of genes to be utilized in the genetic improvement of jute using genetic engineering.

The cDNA library of C. *capsularis* constructed by Islam et al. contained 106 primary clones comprising about 90% recombinants, and the average insert size as determined was 100 to 500 bp (Islam et al., 2005). Taliaferro et al. constructed a cDNA library of C. *capsularis*, which contained a sufficient number of primary clones comprising about 65% recombinants. The average insert size was 150 to 500 bp (Taliaferro et al., 2006). The library constructed by Samanta et al., 2015 using suppression subtractive hybridization resulted in 2,685 expressed sequence tags, which were assembled and clustered into 225 contigs and 231 singletons. It seems that the suppression subtractive hybridization library can result in more ESTs than cDNA library.

In our study, the functions of the encoded proteins from ESTs sequences were classified into six categories based on molecular function, and the most abundant GO terms are energy production and conversion-related genes, carbohydrate transport and metabolism-related genes. Additionally, amino acid transport and metabolism genes are also one important GO term, as well as the translation, ribosomal structure and biogenesis genes. While the other two types of genes, inorganic ion and lipid transport and metabolism genes, are the least abundant GO terms. Our results are similar with Islam's. According to the report of Islam, based on similar homology resulted the partial cDNAs encode proteins, including ribosomal protein, transport protein and chloroplast inner membrane protein. Taliaferro et al. also found several significant sequences of jute ESTs by analyzing the library, including those of the 60S acidic ribosomal protein and the Class I chitinase (Taliaferro et al., 2006). Moreover, our study indicated unigenes of *C. capsularis* have highest homology to *P. trichocarpa*, and which also have higher homology to *R. communis*, *C. olitorius* and *V. vinifera.* The results are different with Wazni et al., who reported the homology was maximum between black jute (*olitorius*) and cotton followed by citrus, grapevine, tobacco and arabidopsis (Wazni et al., 2007), which illustrates the differences between black and white jute. Above results would be a potential resource for comprehensive genomic studies in *Corchurus* species. The collection of ESTs presented in this study should prove useful tools for identifying *C. capsularis* homologues to important genes from other organisms. Nevertheless, the EST data presented here were limited. High and medium abundance expressed genes have a lower proportion of 39.35% relatively, while which often represent the specific characteristics or functions of cell or tissue. Whatever, the initial data of *C. capsularis* sequences will undoubtedly provide a foundation for future research.

In conclusion, this study presents the construction of cDNA library and analysis of ESTs from leaves of *C. capsularis*. The ultimate objective of the present study was to establish a database on white jute genome analysis and repository of ESTs and cDNA, which will be freely available to jute researchers all over the world to identify new genes and supply an effective alternative strategy for functional genomics. Further analysis will involve screening of the current library with probes of these expressed genes. Moreover, many of the sequences need to be isolated by cloning the cDNA ends. Future study aims at collecting suitable material from other parts of the plant such as young stem, bark tissue to clone genes controlling many important traits of agronomic importance including those for regulating lignin biosynthesis.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Toxicity assessment of modified Cry1Ac1 proteins and genetically modified insect-resistant Agb0101 rice

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Insect-resistant Agb0101 rice was recently developed by modifying the *cry1ac1* gene by changing codon usage changes relative to the native truncated *cry1ac1* gene. To assess the toxicity of genetically modified Agb0101 rice, we conducted bioinfomational comparisons of the amino acid sequences that are not similar to known toxic proteins. Sufficient quantities of mCry1Ac1 protein were produced in *Escherichia coli* for *in vitro* evaluation and animal study. We compared the amino acid sequences and molecular mass. There have the same amino acid sequences and molecular masses after purifying the modified Cry1Ac1 (mCry1Ac1) protein from highly expressed bacteria and genetically modified rice were identical. We also investigated the acute and 90-days oral toxicities. No adverse effects were observed in mice following acute oral exposure to 2,000 mg/ kg body weight mCry1Ac1 protein of body weight and 90 days oral exposure to Agb0101. These results indicate that mCry1Ac1 proteins and Agb0101 rice demonstrate no adverse effects in these tests when applied via gavage and feed, respectively.

Key words: Modified Cry1Ac1, food safety assessment, toxicity, insect- resistant rice Agb0101.

INTRODUCTION

Genetically modified (GM) crops are becoming an increasingly important feature of the agricultural land-scapes. In 2013, approximately 175 million hectares of GM crops were planted by 18 million farmers in 27 countries. Due to the unprecedented 100-fold increase between 1996 and 2013, biotech crops are now the

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Abbreviations: **ALP,** alkaline phosphatase; **ALT,** alanine aminotransferase; **AST,** aspartate aminotransferase; **BUN,** urea nitrogen; **TG,** triglyceride; **A/G Ratio,** albumin/globulin ratio; **IP,** inorganic phosphorus.

fastest-adopted crop technology in the history of modern agriculture. Products with insect-resistant traits were the fastest growing group between 2009 and 2013 (James, 2013). Rice (*Oryza sativa*) is an important crop and staple food that used to main energy source in Korea. Not only the major food but also economical impact, Rice production affects all around of Korea economy. In Korea, 1 million people are cultivating rice in about 1 million ha of rice field. Various factors limit rice productivity, including pest which annually destroy 20 to 30% of rice crops (Estruch et al., 1996). Pest managements are very important factor to consider when attempting increasing rice production. In Korea, C*nophalocrocis medinalis guenee* is the one of major problem in rice cultivation especially. Generally, this insect can be managed using several kinds of pesticides (Warren et al., 1997). However, many farmers and consumers want to use less pesticide due to environmental and economic concerns. Insect-resistant rice was developed to prevent pest explosions and seek eco-friendly purposes. Many GM Crops for pest managements are developed and commercialized. Almost 32% of commercial GM crops are insect-resistant due to prevent pest damage. Almost all GM Crops use the gene as commonly known as Bt gene from the bacterium *Bacillus thuringiensis* that code for insecticidal crystal proteins were engineered into plants in the mid-1980s in order to develop the insect-resistant genetically modified plant. Bt corn was the first genetically modified by introducing the *Bt* gene since 1987 to combat the crop damage. Bt products have been used as insecticides for more than 40 years without documented evidence of adverse effects. Additionally, a number of toxicological studies on insecticidal Cry proteins have not identified any safety concerns for using Bt proteins (McClintock et al., 1995).

Genetic modification using insect-resistant genes is one of the most common modifications used to generate transgenic crops, including rice (Bajaj and Mohanty, 2005; Maqbool et al., 2001; Ramesh et al., 2004; Tu et al., 2000; Ye et al., 2003). Commercializing GM rice lags behind other cereals such as maize. One reason is that rice is cultivated more than 100 countries around the world and is a staple for about a half of the world's population; thus its safety must be strictly evaluated prior to its release to the market (Jiao et al., 2010). The *cry1Ac* gene can be isolated from *B. thuringiensis* and encodes the Cry protein, which exhibits toxic effects by forming pores in the cell membrane, thereby injuring epithelial cells in the midgut of insect. The effect is highly specific to target insects such as Lepidoptera and Coleoptera species, but is harmless to plants and mammals including humans (Bravo et al., 2007). We recently developed insect-resistant Agb0101 rice that contains the *mcry1Ac1* gene, a modified synthetic and truncated version of the *cry1Ac1* gene that expresses the same toxic protein with Cry1Ac1. Agb0101 rice contains a single copy of the truncated *cry1Ac* gene, and the toxic protein expressed by this transgene targets the chloroplast (Lee et al., 2009). Furthermore, research has revealed that Agb0101 demonstrates high resistance to rice leaf folder, rice green caterpillar, and rice skipper under laboratory conditions and to rice leaf folder under field conditions (Kim et al., 2009).

This study was performed to assess the toxicity of insect-resistant rice, develop scientific methodologies assess the safety of GM crops. These results could be used to further commercialization of insect-resistant Agb0101 rice. Although, this study described toxicity only, our results could help elucidate the food safety of Agb0101 rice.

MATERIALS AND METHODS

Test materials

The modified c*ry1Ac1* gene (*mcry1Ac1*: GenBank accession no. AY126450) used in this study was derived from the truncated *cry1Ac1* gene (GenBank accession no. AAA22551). This *mcry1Ac1* gene encodes the same amino acid sequences with the truncated Cry1Ac1 protein. For transformation into rice, some nucleotides were changed for optimal use in plants. Because the expression level of the mCry1Ac1 protein is extremely low in transgenic Agb0101 rice (typically less than 100 mg/kg), it is impractical for use in animal studies. Therefore, we conducted safety assessments using mCry1Ac1 protein that was produced in *Escherichia coli*. Proper characterization of the equivalence between recombinant and rice expressed protein is a necessary pre-requisite for use in safety evaluations of specific transgenic events. Agb0101 and control rice (Nak-dong) were grown in the experimental field of the National Academy of Agricultural Science (Suwon, Korea). To purify the mCry1Ac1 protein from rice, transgenic rice leaves containing the *mcry1ac1* gene were used as the plant material. Samples were collected, and the leaves were stored at -80°C until use.

Expression and purification of mCry1Ac1 protein from *E. coli*

The mCry1Ac1 proteins were produced and characterized. The pMAL-p2g/mcry1ac1 vector (BioLabs Inc., USA) was used to express the mCry1Ac1 protein. *mCry1Ac1* referred to the modified *cry1ac1* gene in order to be expressed in *E. coli* strain BL21 CodonPlus (DE3) RIPL (Stratagene, La Jolla, CA) as a fusion protein containing a maltose binding protein(MBP) tag and was purified using immobilized amylase resin chromatography. The MBP tag was cleaved from the affinity purified protein with cysteine protease from Tobacco Etch virus (TEV). And the fusion tag and TEV were removed by dialysis. For the acute toxicity study, the protein was lyophilized, mixed and stored at −80°C. The purity of the total protein was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and scanning densitometry. The concentration of the total protein was determined using the Bradford method. The identities of both lots of proteins were characterized using amino acid analysis and western blot analysis using a proprietary mCry1Ac1- specific antibody.

Using bioinformatic analysis to determine similarities between toxic proteins and the mCry1Ac1 protein

Sequence similarity searches were conducted on the amino acid sequence of the mCry1Ac1 protein using the BLASTP algorithm of the NCBI protein database. Sequence similarities were manually

Table 1. Study design of feed composition for the sub-chronic study.

Ingredient (%)		G1	G2	G3	G4
No. of animals	Male	10	10	10	10
	Female	10	10	10	10
Agb0101 rice		0	0	5	20
Nak-dong rice		0	20	0	0
Feed		100	80	95	80
Total		100	100	100	100

inspected manually to identify known toxic proteins. Generally, a st atistically significant sequence similarity requires a match with an E score of <0.01; however, a threshold E score 1.0 provides greater assurance that proteins with even limited similarities will not be overlooked. The BLOSUM62 scoring matrix was used, low complexity filtering was turned off, and the number of alignments returned was set to a maximum value of 1,000 proteins.

Protein sequencing and In-gel trypsin digestion and protein identification by MS/MS

The mCry1Ac1 protein was electroporated and transferred to PVDF membranes. Targeted band were cut and amino acid sequencing was carried out by Edman Sequencer ABI494. MALDI-TOF mass experiments were carried out according to Sun et al. (2007), with minor modifications. Protein bands with mCry1Ac1 were carefully cut out from CBB R250-stained gels and subjected to in-gel trypsin digestion according to Sun et al. (2007), with minor modifications. MALDI-TOF/TOF-GPS Explorer™ software version 3.6 (Applied Biosystems) was used to create and search files with the MASCOT search program for peptide and protein identification.

Acute toxicity assessments of the mCry1Ac1 protein in ICR mice

Acute toxicity studies were conducted in accordance with OECD guidelines (OECD, 2001) and the Laboratory animal Act by Korea Food and Drug Administration (KFDA). 60 ICR mice (3 weeks old, 18 to 22 g) were obtained from Hanlim Animal Experiment Institute, (Hwasung, Korea). After 5 day acclimatization period, the mice were randomly divided into 3 groups. Each group was related to mCry1Ac, Bovine serum albumin (BSA) and water as control. Each group contained 20 mice / group (10 males and 10 females per treatment). All mice were kept in stainless steel wire cages (2/cage) at 21 to 23°C, relative humidity 40 to 60%, 15 air change times per hour, and electric lights were turned on from 9 AM to 9 PM. Mice were allowed free access to both food and water. The mCry1Ac1 protein was dissolved in distilled water to 95% purity, and the mCry1Ac1 protein concentration was adjusted to 250 mg/mL. Each mouse received about 0.2 mL/kg protein (2,000 mg/kg mCry1Ac1 protein for each mouse) by gavage on the first day. BSA was used as the negative control and water was used as the blank control. Mice were given basal diet and tap water and observed for 14 days for any signs of morbidity or mortality. During the experimental period all animals were inspected twice daily (cage side). At study completion day 15, all animals were anaesthetized by carbon dioxide inhalation and sacrificed by exsanguinations for the subsequent gross and histopathological examinations.

Subchronic toxicity of Agb0101 rice in SPF rats

Subchronic toxicity study was conducted in accordance with OECD guidelines (OECD, 1998) and the Laboratory animal Act by KFDA. 80 SPF rats (40 male and 40 female) were obtained from Orient Co. (Kapyung, Korea). All rats were 5 weeks old at study initiation. Following a 7 day acclimatization period, they were randomly divided into 4 groups, 20 mice/ group (10 males and 10 females per treatment). All diets were administered to rats for 90 consecutive days. Animals were housed pair wise in stainless steel wire cages at 23 ± 2°C, relative humidity 55 ± 5%, 10 to 15 air change per hour and electric light with 200 to 300 lux from 9 AM to 9 PM. Rats were allowed free access to both food and water. Insect resistant Agb0101 rice was mixed with the basal diet in order to take into account nutritional balance. We made 4 kinds of feed for this experiment. The composition of the feed administrated to each group is listed in Table 1. Diets were identically adjusted to assure an adequate supply of macronutrients and vitamins after substitution with 60% rice, but no adjustments were made to balance differences in the constitution of the rice (as observed by the compositional/chemical analyses). Rats were allowed free access to both food and water (KFDA, 2007).

Serum biochemistry and haematology

All animals were fasted overnight to minimize fluctuations in the measured parameters. Blood samples were taken from the eyeball veniplex and stabilized using heparin. The following biochemical parameters were measured: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea nitrogen (BUN), triglyceride (TG), albumin/globulin ratio (A/G ratio), inorganic phosphorus (IP), sodium, potassium, cholesterol, protein, albumin, creatinine and glucose. All analyses on blood plasma were performed on a Express plus (Bayer Diagnostics Inc., USA). Blood samples used to assess the hematology characteristics were stabilized using EDTA. The following characteristics were assessed using the Baker system 9118 Hematology Analyzer (Biochem Immunosystems Inc., Allentown, PA). White blood cells, Red blood cells, hemoglobin concentration, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration and platelet count. The differential count distinguishes between basophils, eosinophils, lymphocytes, monocytes and neutrophils.

Organ weights, gross necropsy and histopathology

Necropsy was performed and the following organs were sequentially excised: brain, thymus, heart, lungs, liver, spleen, stomach, duodenum, adrenals, kidneys, ovaries, and testes. The brain, heart, liver, spleen, lungs, kidneys, thymus, adrenals, ovaries and testes

were immediately weighed, and the stomach, duodenum, kidneys and liver were immediately fixed in 4% buffered formaldehyde for histological processing. Tissue samples were embedded in paraffin, and sectioned to 3 to 5 µm thick, and then stained with standard hematoxylin–eosin for light microscopy.

Statistical analysis

Statistical comparisons were designed to determine if differences in the mentioned response variables between groups could be attributed to the mCry1Ac1 protein and Agb0101 rice. Data obtained from the mCry1Ac1 protein groups were compared with the values from the vehicle and BSA control groups. Similarly, each Agb0101-treated group was compared with the values from the control groups. Homogeneity variance was analyzed using one-way analysis of variance (ANOVA) using Statistical Product and Service Solutions (SPSS) v12.0 (SPSS Inc., Chicago, IL). Differences were considered significant when $p<0.05$ and then a step-down analysis was conducted using least squares differences (LSD).

RESULTS

BlastP comparison of the mCry1Ac1 proteins to other toxic proteins

BLASTP similarity searches were conducted using the amino acid sequence of mCry1Ac1 in order to identify the 1000 closest matches. The highest E score returned was 0.85, confirming that sequences with limited similarity were not overlooked by the search. 95 of the accessions returned by the searches demonstrated complete significance (E = 0) and represent very closely related Cry proteins from various bacterial species. A total of 827 other sequences were identified as Cry proteins from various bacterial species. The remaining 173 hits represented a variety of proteins that are all functionally related by the possession of one or more well characterized conserved thiamine pyrophosphate binding domains. No information is available on the toxicity of these proteins returned by the BLASTP search identified similarities to proteins known toxic. Assessing the safety of a novel protein requires determining its amino acid sequence similarity with known toxic proteins that have potential safety concerns. Accordingly, amino acid sequences were compared as part of the current food safety decision tree strategy as recommended by FAO/WHO (2001) and the CODEX (2003). Amino acid sequence similarities between the mCry1Ac1 protein and known toxic proteins were determined according to published guidelines (CODEX, 2003). The results of the *in silico* analysis revealed no evidence for similarities between mCry1Ac1 and any known toxic protein.

Isolation and characterization of heterologously produced mCry1Ac1 protein

The recombinant mCry1Ac1 protein was expressed in *E. coli* and purified as a soluble protein. The mCry1Ac1 protein migrated as a major band and demonstrated a molecular weight of approximately 66 kDa according to our SDS–PAGE analysis. The purity of the mCry1Ac1 protein was greater than 95% according to densitometry analysis which was performed the SDS–PAGE analysis (Figure 1). After blotting to PVDF membrane, we performed N-terminal sequencing by Edman degradation. The N-terminal sequence of the analyzed mCry1Ac1 protein was identical to deduced amino acid sequence of mCry1Ac1. MALDI-TOF MS analysis confirmed 71% sequence coverage for the mCry1Ac1 protein (data not shown).

Acute toxicity of mCry1Ac1 protein

The acute toxicity of the mCry1Ac1 protein was assessed in mice following the oral administration of the purified heterologously expressed protein at 2,000 mg/kg body weight of the test substance (corresponding to approximately 2,000 mg/kg of body weight of mCry1Ac1 protein) via oral gavage. Control groups were administered either vehicle (that is, water) alone or BSA at 2,000 mg/kg body weight. All mice survived the study period and no clinical signs of systemic toxicity were observed in any treatment groups (data not shown). All mice in all treatment groups gained weight relative to day 0 of dosing (Table 2), and no gross lesions were present in any of the mice at necropsy, thereby indicating that the mCry1Ac1 protein was not acutely toxic. The mCryAC1 protein demonstrates no special toxicity according to our acute toxicity tests. We did not observe animal death and special symptoms at 2,000 mg/Kg body weight.

Subchronic toxicity of genetically modified rice Agb0101

Mortality and clinical signs

There was no instance of treatment-related mortality in the animals treated with Agb0101 rice during this study. No significant clinical signs were observed in any other group.

Body weight and feed consumption

No reliable differences in body weight (Figure 2), feed consumption, weight gains, or food efficiency were observed between rats in the Agb0101-treated groups and those in the related control group (that is, rat that received Nak-dong rice). Also, no reliable changes in consumption between treated groups were observed during the test periods (Figure 3).

Hematology

As shown in Table 3, no significant changes were observed

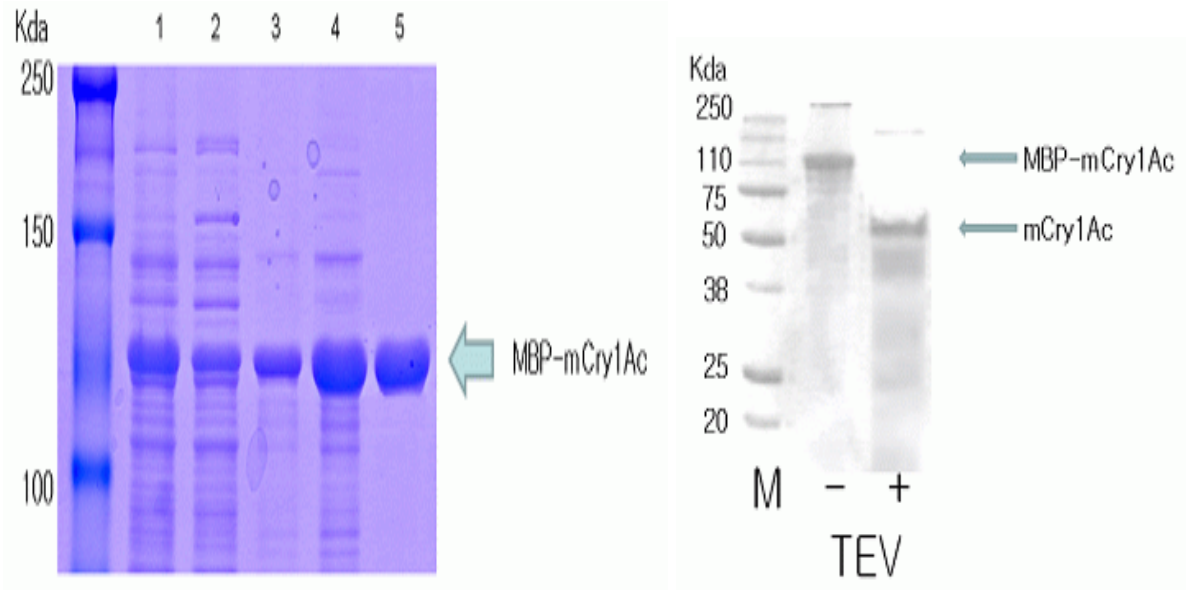

Figure 1. Purification of microbial mCry1Ac1 protein. (A). SDS-PAGE analysis of microbial mCry1Ac1 protein. Lane 1 contains total cell extract; Lane 2 contains soluble fraction of cell extract; Lane 3 contains amylase resin eluted fraction; Lane 4 contains concentrate of amylase resin eluted fraction; Lane 5 contains anion-exchange chromatography eluted fraction. (B). The Cleavage of MBP-mCry1Ac1 protein. Lane M contains molecular weight marker; Lane – contains MBP-mCry1Ac1 protein without TEV protease; Lane + contains mCry1Ac1 protein with TEV protease.

Table 2. Body weights changes of mice from acute toxicity study with mCry1Ac1 protein.

Materials	Female			Male		
	0	7	14	0	7	14
m Cry1Ac1	22.1±0.79	24.6±1.36	26.8±1.41	31.5±1.34	34.3±1.77	37.6±1.96
BSA	22.7±1.66	25.5±1.93	26.6±1.95	31.6±1.83	35.0±3.23	38.4±3.35
Water	22.5±1.51	24.5±1.73	27.1±1.88	30.6±1.53	34.2±2.32	37.4±2.83

in most of the hematology response variables between groups that consumed the different diets. However, certain hematology variables, such as reticulo-cytes in the male rats in the G4 group demonstrated significant decrease in comparison with G1 whereas female in the G3 and G4 groups demonstrated significant increases in comparison with G1, G2 groups. Reticulocytes are a known indicator of anemia, but related variables like red blood cells, hemoglobin, and hematocrit did not significantly changed in either males or females. These results indicate that the reticulocyte changes in males and females were not cause by Agb0101 rice.

Serum biochemistry

No differences were observed in most of the values of the serum response variables between the groups consumed different diets (Table 4). However, some differences were observed in total protein and some ion parameters. Total protein demonstrated a significant decrease among female in the G4 and G1 groups. However, this value difference was not observed in the G2 group females. Calcium ion also statistically and significantly decreases in G3 and G4 females, but not in males, though this finding was not significantly between the G1 and G4 groups. Chloride ion decreased in G3 and G4 males in comparison with G1 males, but increased in G4 female comparison with G1 females. These changes were not consistently demonstrated in males and females, and the increases/decreases were inconsistent. Therefore, the observed differences in these parameters are attributed to background variability and sporadic deviation. All differences are within the normal ranges for rats. Therefore, they are not considered related to Agb0101 rice.

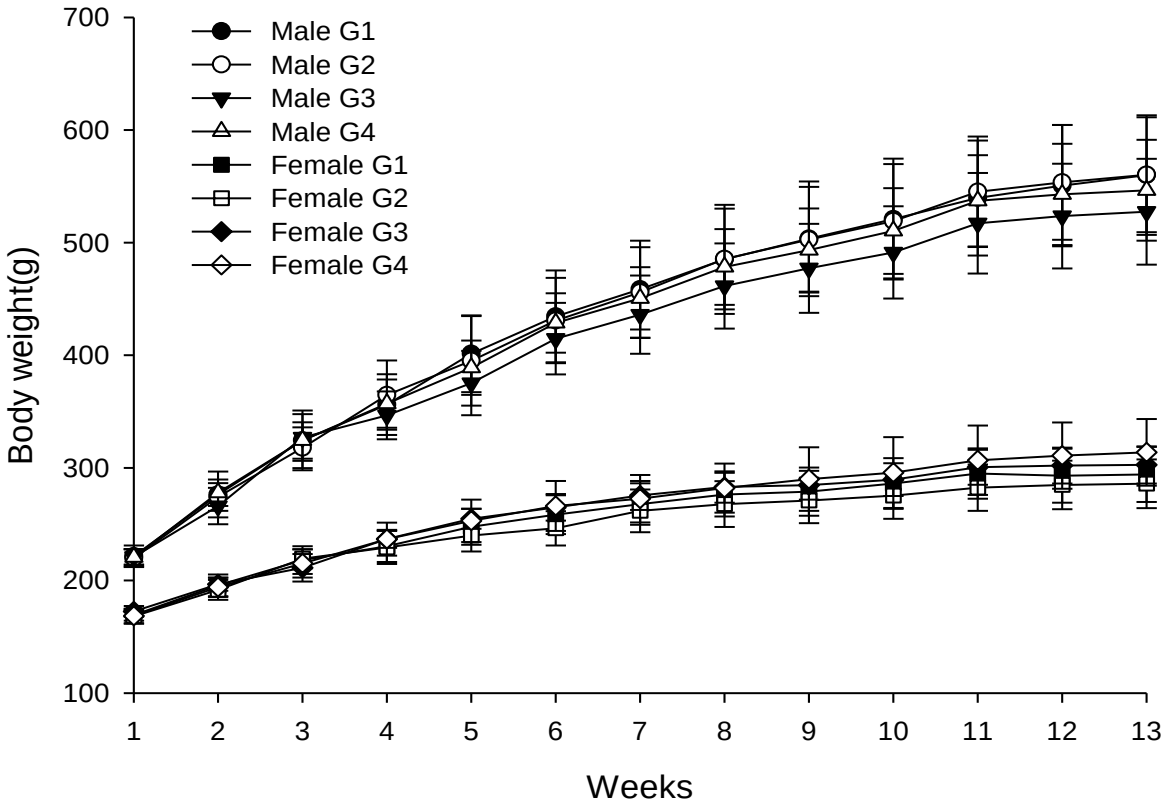

Figure 2. Body weight changes of rats orally treated with Agb0101 rice for 90 days.

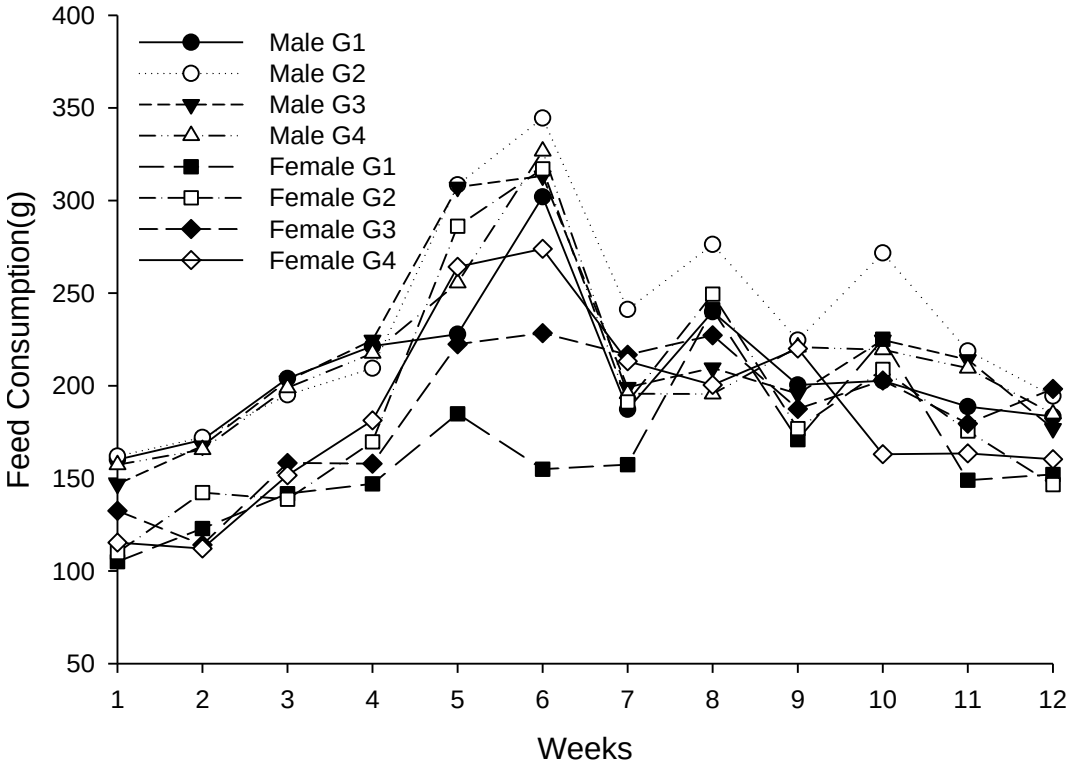

Figure 3. Feed consumption of rats orally treated with Agb0101 rice for 90 days.

Table 3. Analysis of Blood components contents treated with Agb0101 rice.

Sex	Blood components Group (n=10)	G 1	G 2	G 3	G 4
	Basophils(%)	0.06±0.03	0.03±0.02	0.04±0.02	0.03±0.02
	Eosinophils (%)	0.42±0.27	0.35±0.23	0.44±0.21	0.53±0.34
	Hematocrit (%)	42.54±3.61	42.53±2.62	42.08±3.90	44.55±3.94
	Hemoglobin (g/dl)	16.42±0.74	16.57±1.04	16.48±0.92	17.06±0.61
	Lymphocytes (%)	47.35±3.72	48.45±5.83	46.44±8.04	43.54±6.41
	Monocytes (%)	9.92±1.71	11.87±3.05	11.32±4.64	14.35±4.21
	Neutrophils (%)	37.28±3.49	38.83±4.61	41.75±6.01	41.51±6.14
Male	Red Blood Cell (10^6/mm^3)	7.82±1.06	7.82±0.53	8.01±0.59	8.37±0.64
	Reticulocyte (%)	0.93±0.21	0.91±0.22	0.82±0.18	0.69^{a}±0.10
	White Blood Cell (10^3/mm^3)	7.67±2.29	8.17±2.09	7.88±2.46	5.92±1.89
	Activated Partial Thromboplastin Time (sec)	23.74±2.87	22.65±2.10	22.76±1.75	23.63±3.41
	Prothrombin Time (sec)	20.58±3.26	19.82±1.74	18.98±1.45	19.64±3.24
	Mean Corpuscular Hemoglobin (pg)	21.13±1.37	21.61±0.91	20.84±2.82	21.49±2.36
	Mean Corpuscular Hemoglobin Concentration (g/dl)	38.39±2.14	39.07±1.84	39.37±2.45	38.44±2.38
	Mean Corpuscular Volume (fL)	53.37±3.45	54.56±2.82	52.63±2.43	52.81±2.84
	Basophils (%)	0.03±0.02	0.03±0.02	0.05±0.03	0.04±0.02
	Eosinophils (%)	0.76±0.44	0.61±0.25	0.63±0.23	0.71±0.47
	Hematocrit (%)	42.58±4.32	42.85±5.11	39.83±6.87	44.35±5.14
	Hemoglobin (g/dl)	15.28±0.72	15.83±1.21	15.65±0.79	15.32±1.59
	Lymphocytes (%)	53.82±7.91	48.94±7.89	55.64±9.85	50.97±9.52
	Monocytes (%)	10.03±2.66	11.48±1.77	10.69±2.82	11.31±2.26
	Neutrophils (%)	35.44±7.31	39.53±7.92	34.53±9.25	37.08±8. 43
Female	Red Blood Cell (10^6/mm^3)	7.51±1.05	7.65±0.88	7.39±1.32	7.87±0.78
	Reticulocyte (%)	0.74±0.21	0.81±0.22	$1.28^{a,b}$±0.29	$1.31^{a,b}$±0.25
	White Blood Cell (10^3/mm^3)	3.68±1.38	3.53±1.51	3.25±0.75	4.18±1.29
	Activated Partial Thromboplastin Time (sec)	21.57±1.46	21.13±1.30	22.03±1.85	21.28±1.18
	Prothrombin Time(sec)	15.69±1.23	15.84±0.67	16.34±0.61	16.37±0.60
	Mean Corpuscular Hemoglobin (pg)	21.11±1.75	21.35±1.94	22.47±5.63	19.85±1.55
	Mean Corpuscular Hemoglobin Concentration (g/dl)	36.78±2.29	38.89±3.69	40.16±9.71	35.39±3.75
	Mean Corpuscular Volume (fL)	55.91±2.05	55.78±2.26	54.86±1.88	55.33±2.96

Significant differences as compared with G1: $^{a}P < 0.05$. Significant differences as compared with G2 : $^{b}P < 0.05$.

Necropsy findings and organ weight

There were no gross indications of adverse effect in any rat organs on necropsy. No statistically significant differences were observed in the mean relative organ weights between the different treatment groups and the control group (Table 5). Additionally, no adverse effects were noted on histopathological observation (Table 6). Insect-resistant Agb0101 rice was mixed with normal feed at two different concentrations, 5 and 20%. The amounts of protein incorporated into the diet of each groups were 92 and 132 mg/Kg, respectively. As control groups, normal Nak-dong rice also mixed with normal feed into the diet at 20%. Over the course of the in-life phase of this study, no statistically significant differences in body weights (Figure 2) or feed consumption were observed between the control groups and the groups that consumed Agb0101 rice-containing diets (Figure 3). The consumed doses of mCry1Ac1 protein averaged 0.42 and 1.62 mg/kg body weight/day in males and 0.3 and 1.3 mg/kg body weight/day in females that calculate from the results of Kim et al. (2013).

DISCUSSION

This study describes our toxicity assessment that was

Table 4. Biochemical values of SD rats orally treated with Agb0101 for 90 days.

Sex	Biochemical values Group (n=10)	G 1	G 2	G 3	G 4
	T-Protein (g/dl)	6.01±0.17	6.09±0.25	6.16±0.22	6.09±0.20
	Albumin (g/dl)	2.29±0.16	2.34±0.08	2.28±0.12	2.35±0.11
	A/G ratio	0.62±0.26	0.62±0.13	0.59±0.07	0.63±0.09
	ALP (IU/L)	203.2±44.38	208.1±38.57	202.6±38.32	186.2±39.15
	AST (IU/L)	101.7±29.47	98.9±18.38	97.8±17.89	103.5±14.88
	ALT (IU/L)	38.9±5.65	39.5±6.23	39.7±8.91	38.5±6.83
	BUN (mg/dl)	14.76±2.38	15.06±1.79	16.94±2.41	17.16±2.38
	Cholesterol (mg/dl)	68.6±15.44	71.4±16.06	72.5±10.84	70.5±7.79
Male	Creatinine (mg/dl)	0.54±0.06	0.55±0.06	0.56±0.06	0.56±0.08
	Glucose (mg/dl)	164.1±36.28	170.0±17.68	165.2±34.92	165.9±19.93
	TG (mg/dl)	83.83±39.65	84.10±36.92	82.30±26.81	73.40±19.76
	Calcium (mg/dl)	10.19±0.62	9.96±0.61	10.84±0.55	9.98±0.43
	Cl-(mmol/L)	124.6±5.56	117.3±3.02	114.3[a]±3.07	109.9[a,b]±1.17
	IP (mg/dl)	6.13±0.85	6.24±0.67	5.99±0.49	6.13±0.54
	K+(mmol/L)	4.32±0.83	4.33±0.56	4.46±0.45	4.53±0.47
	Na+(mmol/L)	145.1±1.45	143.8±1.94	146.3±1.62	145.7±1.46
	T-Protein (g/dl)	7.27±0.23	6.92±0.29	6.95±0.32	6.78[a]±0.28
	Albumin (g/dl)	3.19±0.21	3.15±0.33	3.19±0.17	2.96±0.33
	A/G ratio	0.78±0.09	0.84±0.11	0.85±0.08	0.77±0.13
	ALP (IU/L)	103.1±45.43	98.7±20.21	93.8±24.55	96.5±20.19
	AST (IU/L)	123.9±31.14	99.1±24.88	93.6±26.31	101.5±27.65
	ALT (IU/L)	41.8±19.23	36.7±22.44	39.5±10.86	38.9±20.42
	BUN (mg/dl)	16.45±2.32	17.14±2.43	18.34±3.97	17.18±1.81
	Cholesterol (mg/dl)	85.1±12.77	84.4±17.30	89.2±19.31	87.1±13.11
Female	Creatinine (mg/dl)	0.63±0.11	0.67±0.06	0.65±0.13	0.64±0.09
	Glucose (mg/dl)	139.4±16.22	140.5±15.37	142.3±11.19	139.3±11.27
	TG (mg/dl)	11.6±6.86	10.3±3.54	9.7±3.85	12.5±11.06
	Calcium (mg/dl)	11.90[b]±0.23	10.87[a]±0.16	10.11[a]±0.17	10.24[a]±0.16
	Cl-(mmol/L)	109.1±2.03	108.5±1.47	108.1±3.08	124.2 [a,b]±4.93
	IP (mg/dl)	5.81±1.48	5.55±1.13	5.72±0.52	5.65±0.83
	K+(mmol/L)	3.97±0.45	3.93±0.33	4.03±0.29	3.99±0.23
	Na+(mmol/L)	145.9±1.25	145.9±1.61	146.1±1.78	146.3±1.32

Significant differences as compared with G 1 : [a]$P < 0.05$. Significant differences as compared with G 2 : [b]$P < 0.05$.

conducted on the mCry1Ac1 protein and Agb0101 rice. This assessment evaluates the potential toxicity of transgenic proteins using weight-of-evidence and tiered approaches, respectively (CODEX, 2003; EC 2003). No evidence for potential toxicity was identified for the *mcry1Ac1* gene according to the components of a first tier. The bt gene was obtained from *Bacillus sp.,* which has a long history of safe use in agricultural pesticides. Rice also has a long safe history as a common component of the human diet. Human exposure to the mCry1Ac1 protein is most likely extremely low because it is present at low concentrations in the entire genetically modified Agb0101 rice plant. The effects of the mCry1Ac1 protein determined by the 90-day toxicity study should be considered in comparison to the concentration determined in rice seeds obtained from genetically modified insect-resistant Agb0101 rice as determined using mCry1Ac1-specific ELISA (0.2 mg/Kg mCry1Ac1 protein in dry tissue). The average human (average body weight 60 kg) would need to consume about 60 kg/day of grain expressing the mCry1Ac1 protein to approximate the same daily dose consumed by the high dose group enrolled

Table 5. Organ weight in treated rat.

Sex	Organ weight group (n=10)	G 1	G 2	G 3	G 4
	Terminal body weight	538.59±50.02	555.67±50.38	522.95±48.67	543.07±46.14
	Adrenal gland	0.029±0.004	0.028±0.005	0.026±0.004	0.029±0.005
	Brain	2.11±0.08	2.12±0.06	2.12±0.09	2.12±0.08
	Epididymis	0.73±0.08	0.74±0.11	0.73±0.06	0.75±0.06
Male	Heart	1.67±0.15	1.68±0.18	1.71±0.13	1.72±0.14
	Kidney	1.53±0.15	1.52±0.12	1.51±0.17	1.55±0.16
	Liver	12.58±1.61	12.77±1.62	12.94±1.94	12.42±1.33
	Pituitary gland	0.014±0.002	0.013±0.002	0.014±0.003	0.016±0.005
	Spleen	0.73±0.12	0.74±0.25	0.73±0.19	0.75±0.16
	Testis	1.73±0.22	1.69±0.24	1.68±0.21	1.72±0.18
	Thymus	0.39±0.11	0.41±0.10	0.38±0.10	0.39±0.13
	Terminal body weight	291.72±25.41	289.71±25.64	299.38±26.40	301.62±28.89
	Adrenal gland	0.034±0.007	0.034±0.006	0.033±0.007	0.034±0.005
	Brain	2.01±0.10	2.04±0.10	2.03±0.09	2.01±0.09
	Heart	1.17±0.21	1.17±0.26	1.09±0.14	1.15±0.21
	Kidney	0.91±0.13	0.89±0.13	0.88±0.14	0.94±0.15
Female	Liver	7.45±0.84	7.25±0.69	7.35±0.87	7.55±0.86
	Pituitary gland	0.020±0.004	0.019±0.004	0.018±0.005	0.017±0.007
	Spleen	0.51±0.08	0.49±0.06	0.48±0.07	0.50±0.06
	Testis	0.060±0.013	0.062±0.012	0.057±0.014	0.062±0.010
	Thymus	0.33±0.08	0.30±0.07	0.31±0.06	0.32±0.04

Table 6. Histopathological findings of rats treated with GM rice for 90 days.

Histopathological finding	Sex	Male		Female	
	Group(n=10)	G1	G4	G 1	G4
Liver	Portal inflammatory cells	9(1)	10(0)	10(0)	10(0)
	Centrilobular apoptosis/vacuolation	10(0)	10(0)	10(0)	10(0)
	Medullary mineralization	10(0)	10(0)	10(0)	10(0)
	Periportal vacuolation	10(0)	10(0)	10(0)	10(0)
	Medullary mineralization	10(0)	10(0)	10(0)	9(1)
	Parenchymal inflammatory cells	10(0)	10(0)	10(0)	9(1)
	Cortical scarring and inflammatory cells	10(0)	10(0)	10(0)	9(1)
Kidney	Papillary mineralization	9(1)	10(0)	10(0)	10(0)
	Inflammatory cells	9(1)	9(1)	10(0)	9(1)
	Cortical vacuolation	10(0)	10(0)	10(0)	10(0)
	Basophilia	10(0)	10(0)	10(0)	10(0)
Adrenal gland	Cortical hypertrophy	9(1)	9(1)	10(0)	10(0)
	Cortical vacuolation	10(0)	6(4)	10(0)	9(1)
Heart	Myocardial inflammation	8(2)	9(1)	9(1)	10(0)
	Endocardial inflammation	10(0)	10(0)	10(0)	10(0)
	Myocardial fibrosis & inflammation	10(0)	10(0)	10(0)	10(0)
	Myocardial necrosis	10(0)	9(1)	10(0)	10(0)

Table 6. Contd.

	Myocardial hemorrhage	10(0)	9(1)	10(0)	9(1)
Lung	Alveolar macrophage	9(1)	9(1)	10(0)	10(0)
	Pneumonitis	9(1)	9(1)	10(0)	10(0)
	Perivascular inflammatory cells	8(2)	10(0)	8(2)	10(0)
	Alveolar osseous metaplasia	9(1)	9(1)	10(0)	10(0)
	Prominent BALT	9(1)	8(2)	8(2)	10(0)
	Alveolar hemorrhage	10(0)	10(0)	10(0)	10(0)
	Pigmented macrophage	10(0)	10(0)	10(0)	10(0)
Spleen	Extramedullary hematopoiesis	8(2)	6(4)	8(2)	6(4)
	Subcapsular vacuolation	9(1)	10(0)	10(0)	10(0)
	Hemosiderosis	10(0)	10(0)	10(0)	10(0)
Epididymis/ Prostate gland	Interstitial infiltration cells	9(1)	10(0)		
	Prostatitis	9(1)	9(1)		
	Interstitial inflammatory cells	9(1)	10(0)		
Uterus	Luminal dilatation			7(3)	8(2)
	Endometrial glandular dilatation			10(0)	10(0)
Thymus	Involution / atrophy	9(1)	10(0)	10(0)	8(2)
	Cortical apoptosis	10(1)	10(0)	10(0)	10(0)
	Cystcs	10(0)	10(0)	10(0)	10(0)
Thyroid gland	Microfollicles	9(1)	10(0)	10(0)	10(0)
	Follicular cell hypertrophy	10(0)	10(0)	10(0)	10(0)
Pancreas	Focal acinar atrophy	9(1)	10(0)	10(0)	10(0)
	Fat replacement	9(1)	10(0)	10(0)	10(0)
	Acinar apoptosis	7(3)	8(2)	9(1)	10(0)
	Inflammatory cells	10(0)	10(0)	10(0)	10(0)
Stomach	Submucosal inflammation	9(1)	10(0)	10(0)	9(1)
	Squamous cyst	9(1)	10(0)	10(0)	10(0)
	Ulceration-non glandular region	10(0)	10(0)	9(1)	10(0)
Urinary bladder	Luminal dilatation	10(0)	9(1)	9(1)	10(0)

*Normal (slight malfunction).

in the 90-day toxicity study. This is an extremely conservative estimate of human exposure to the mCry1Ac1 protein because *in vitro* digestion studies indicate that mCry1Ac1 will not be absorbed intact because it will most likely be degraded within the gut.

Dietary proteins are taken in as nutrients and typically demonstrate no relation to toxic effects. However, some proteins do cause acute toxicity (Metcalfe et al., 1996; Sjoblad et al., 1992). For this reason, guidance documents from CODEX and KFDA described procedures for assessing the potential toxicity of transgenic proteins. Most dietary proteins are non-toxic and absorbed for nutritional purposes. However, because some identified proteins are toxic to mammals and other species, new reliable recommendations were developed to assess transgenic proteins that could cause toxicity using a two-tiered approach (Delaney et al., 2008).

The first tier assesses the history of use of the organism from which the gene is obtained. Generally, similarities between known toxic proteins and transgenic proteins can be determined using bioinformatical analysis, mechanism of action of the protein, *in vitro* stability to digestive enzymes were major research process (Delaney et al., 2008). A second tier analysis may be conducted if there have harmful results. Elements within this tier include acute toxicity studies that use purified transgenic proteins and 90-days oral toxicity (sub-chronic) studies with genetically modified organisms

as requested by regulatory authorities.

Conclusion

Our present data demonstrate the safety of the mCry1Ac1 protein and insect-resistant Agb0101 rice for use in food and feed applications and indicate that the mCry1Ac1 protein and Agb0101 rice presents no risks for adverse health effects when used in the context of agricultural biotechnology. No evidence of toxicity was observed in mice or rats following acute or 90-day oral exposure to heterologously produced mCry1Ac1 protein in Agb0101 rice. Therefore, according to our current study findings, the no-observed-adverse-effect-level (NOAEL) for the mCry1Ac1 protein is more than 10 g/Kg body weight for both male and female mice. Results from these studies further support the use of a tiered approaches that include Tier II studies, such as including hazard characterizations and acute and sub-chronic oral toxicity studies, but do not provide additional information or results that contradict the results of the Tier I studies that do not identify evidence for potential toxicity of the mCry1Ac1 protein.

Conflict of interest

The authors did not declare any conflict of interest.

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Genetic variations between two ecotypes of Egyptian clover by inter-simple sequence repeat (ISSR) techniques

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The inter-simple sequence repeat (ISSR) markers have been used in order to determine genetic variation and relationship between two clover ecotypes. Ten (10) primers for ISSR were used in this study but only six were successful in generating reproducible and reliable amplicons for different types of the Egyptian clover. The results reveal the polymorphism level by ISSR primers. HB10 ISSR-primer was higher than the rest of the ISSR primers in polymorphic 100%. The Fahl monocut ecotype had 29 present bands, 3 absent bands in total of 32 bands; among those there were two unique bands. The multicut ecotype were given different pattern of bands, Gemmiza1 (21 present and 11 absent), Giza6 (21 present and 11 absent) and Serw1; (23 present and 9 absent). There were three unique bands appearance in the two ecotypes. Fahl was given two with HB11 and HB13; the Serw1 multicut cultivar had one unique bands with HB08. Similarity indices among the four Egyptian clover cultivars based on ISSR analysis was estimated and the highest value appeared between Fahl and Gemmiza1 as well as Giza6 and Serw1 followed by Fahl and Serw1. The lowest similarity value appeared between Gemmiza1 and Serw1 followed by Gemmiza1 and Giza6.

Key words: Egyptian clover, molecular marker, Fahl, Gemmiza1, Giza6, Serw1.

INTRODUCTION

Among fodder crops, Egyptian clover (Berseem) has high commercial importance and potential value. Egyptian clover is divided into two ecotypes of recovery status after cutting: first ecotype a single cut mower which cannot renew itself after the cutting while the second ecotype renews itself from five to six times after cutting, including numerous varieties in the Egyptian clover. The cycle of berseem capital is estimated by ten billion US dollars. Berseem is the main forage crops for livestock to produce milk and /or meat in Egypt. Moreover, berseem is the guard on Egyptian soil fertility (Zayed, 2013). Egypt has poor rangeland, although vast areas of more than 10

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million ha exist. The highest total dry matter yield of 15.861 ton ha^{-1} was recorded for Gemmiza1 cultivar. Egypt depends mainly on Egyptian clover as the key forage crop. The cultivated area of berseem in Egypt can reach more than 1.2 million ha in Delta and the Nile Valley annually. There is big competition between berseem and wheat, especially on old land where the productivity is the highest for both crops (FAO, 2010). Although there is a wide gap between the available and the required feed, there is a very rapid development in the animal wealth to meet the high demand for animal products (FAO, 2012).

Four Egyptian clover (*Trifolium alexandrinum* L) cultivars representing two ecotypes were used in the present study. Fahl cultivar is prevalent in whole Egypt and is good for single cut as it has poor regeneration ability, whereas Serw1, Giza6 and Gemmiza1 give 5-6 cuts of good fodder. Techniques based on molecular marker analysis (that is, RFLP, RAPD, ISSR-PCR) may provide more efficient and accurate screening method. Simple sequence repeats comprise short oligonucleotide sequences, two to six bases long, repeated in tandem array, which occur very frequently in eukaryotic genomes (Beckmann and Soller, 1990; Lagercrantz et al., 1993; Tautz and Renz, 1984). They are widely distributed within genomic DNA and are present in both the introns of genes and in non-coding regions. The ISSR-PCR technique uses primers that are complementary to a single SSR and was anchored at the 5′ or 3′ end with a one- to three-base degenerate oligonucleotide (anchor) (Zietkiewicz et al., 1994). This anchor ensures that the primer binds only to one end of a complementary SSR locus. The great number of amplicons generated consists of the region between neighboring and inverted SSRs. As a result, the highly complex banding pattern obtained will often differ greatly between cultivars of the same species. Inter-simple sequence repeats (ISSR) have also been widely utilized for genetic study in the past (Ulloa et al., 2003). The advantage of ISSR over RAPD is its being more reproducible (Fernandez et al., 2002; Greene et al., 2004).

In previous studies, polyacrylamide gel electrophoresis (PAGE) was performed for native and SDS protein and isozyme variations. RAPD was conducted using eight arbitrary 10-mer primers. Combined analysis based on four isozymes, PAGE protein electrophoresis and RAPD analyses revealed high similarity of 0.85 between the cultivars Sakha4 and Gemmiza1, while the lowest similarity (0.53) was observed between Giza6 and Helaly (Tarrad and Zayed, 2009; Zayed et al., 2010).

The Miskawy, Saidi and Fahl ecotypes differ in their morphological yield, regeneration ability after cutting and stage of maximum growth. The Miskawy and Fahl have high inter-varietal variability in terms of green yield, plant height, number of branches and tillers per plant. Helaly is a derivative of Miskawi ecotype (Soliman et al., 2010; Zayed et al., 2010). In the present study, ISSR markers have been used in order to determine genetic variation and relationship between two ecotypes.

Table 1. List of ISSR primers and their nucleotide sequences.

Primer name	Sequence
H8	$(GA)_6GG$
H9	$(GT)_6GG$
H10	$(GA)_6CC$
H11	$(GT)_6CC$
H12	$(CAC)_3GC$
H13	$(GAG)_3GC$

MATERIALS AND METHODS

Plant material

Four Egyptian clover (*Trifolium alexandrinum* L) cultivars representing two ecotypes were used in the present study: one cultivar monocot ecotype (FAh1) and three cultivars multicut ecotype (Serw1, Giza6 and Gemmiza1). Fahl cultivar has 16 chromosome and prevalent in whole Egypt as single cut because it has poor regeneration ability. Serw1cultivar has 16 chromosome, multicut, and cultivated in salinity soil and north of Egypt. Giza6 cultivar has 16 chromosome, multicut, and cultivated in Upper Egypt. Gemmiza1cultivar has 16 chromosome according to Soliman et al. (2010), multicut, and cultivated in all Egypt. Serw1, Giza6 and Gemmiza1 cultivars are distributed in Egypt and can give 5-6 cuts of good fodder. They have higher green fodder yield and has good regeneration ability after cutting.

Genomic DNA extraction and purification

Extraction of total DNA was performed using methods for medicinal and aromatic plants according to Anna et al. (2001). To remove RNA contamination, RNase A (10 mg/ml, Sigma, USA) was added to the DNA solution and incubated at 37°C for 30 min. Estimation of the DNA concentration in different samples was done by measuring optical density at 260 nm according to the following equation: Conc. (µg/ml) = OD_{260} × 50 × dilution factor according to Barbas et al. (2001).

Inter simple sequence repeats (ISSRs)

Ten (10) primers for ISSR were used in the present study but only 6 were successful in generating reproducible and reliable amplicons for different types of Egyptian clover. Names and sequences of the selected primers are shown in Table 1. The amplification reaction was carried out in 25 µl reaction volume containing 1x PCR buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmole primers, 2 units Taq DNA polymerase and 25 ng template DNA. PCR amplification was performed in a Perkin Elmer 2400 thermocycler (Germany). PCR conditions and amplification was programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94°C; each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 40°C for 2 min, and an extension step at 72°C for 2 min, following by extension cycle for 10 min at 72°C in the final cycle.

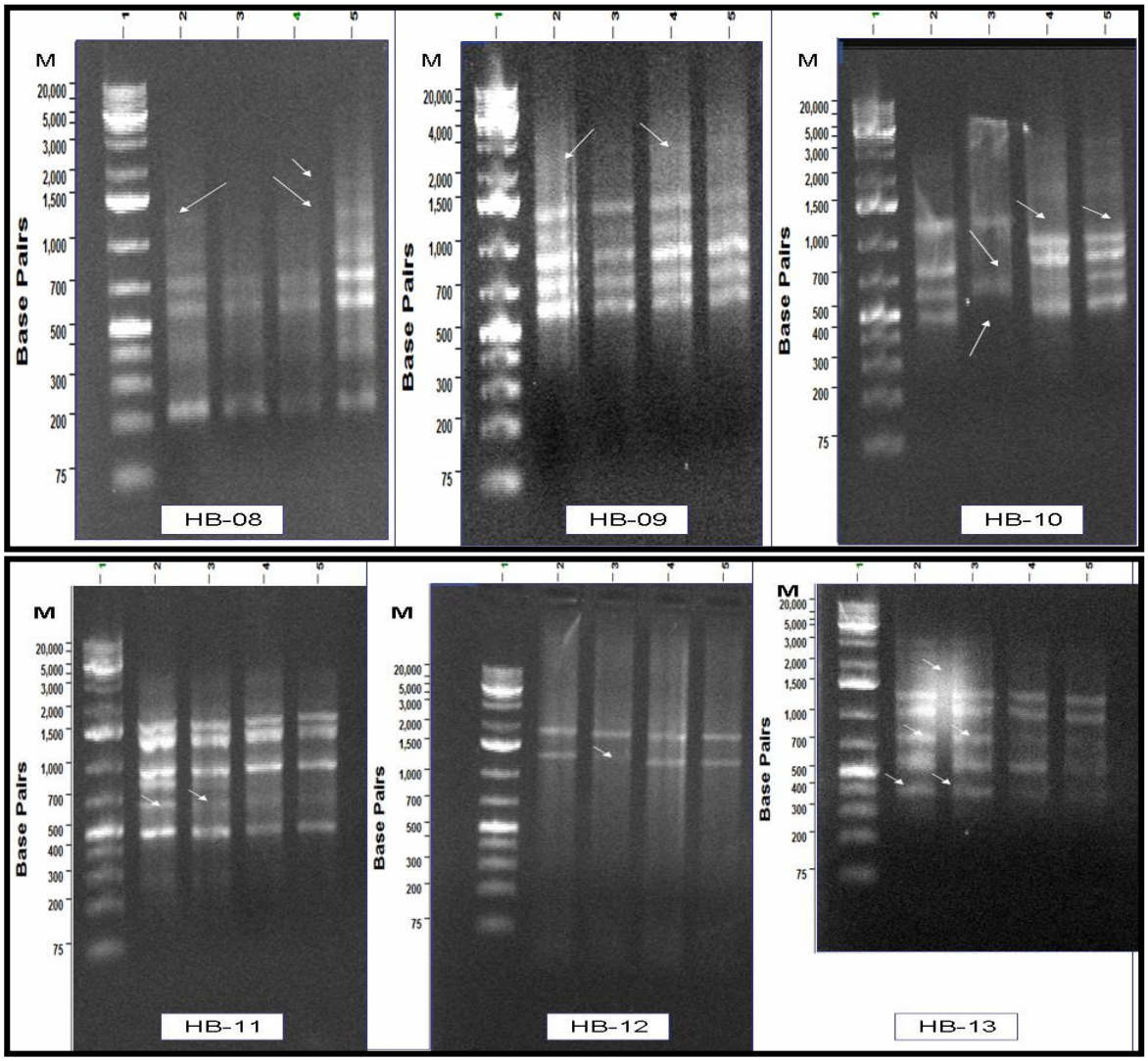

Figure 1. Illustration of ISSR–PCR reaction 6HB primers, HB08, HB09, HB10, HB11, HB12 and HB13 with two ecotypes (Mono and Multicut). 1= 1 kp DNA marker; 2 = Fahl (Monocut); 3=Gemmiza1 (Multicut); 4=Giza6 (Multicut); 5 = Serw1 (Multicut).

Detection of PCR Products

The products of ISSR-based PCR analyses were detected using agarose gel electrophoresis (1.2% in 1X TBE buffer), then stained with Ethidium bromide (0.3 µg/ml) and then visually examined with UV trans-illuminator and photographed using a CCD camera (UVP, UK).

Data analysis

Clear, unambiguous and reproducible bands recovered through different techniques were considered for scoring. Each band was considered a single locus. Data were scored as (1) for the presence and (0) for the absence of a given DNA band. Band size was estimated by comparing with 1-kb ladder (Invitrogen, USA) using Totallab, TL120 1D v2009 (nonlinear Dynamics Ltd, USA). The binary data matrices were entered into the NTSYSpc (Ver. 2.1) and analyzed using qualitative routine to generate similarity coefficient and used to construct a dendrogram using unweighted pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering (SHAN) routine.

RESULTS AND DISCUSSION

Figure 1 shows the DNA banding patterns obtained with ISSR-PCR techniques of the four cultivars Fahl, Gemmiza1, Giza6 and Serw1 using six different ISSR primers. The identified bands that resulted from the ISSR- primer HB8, HB9, HB10, HB11, HB12 and HB13 profile are presented in Figure 1 and Table 2. The polymorphism level shown by ISSR primers confirmed that HB ISSR-primer HB10 was higher than the rest of the ISSR primers (Table 3). The primer HB08 give 7 bands with two ecotypes, one monocut Fahl cultivar and

Table 2. Present and absent bands ISSR-PCR products by HB primers in Four Egyptian clover cultivars.

Primer	MW (bp)	Ecotype 1	Ecotype 2		
		Monocut	Multicut		
		Fahl	Gemmiza1	Giza6	Serw1
HB08					
1	1652	0	0	0	1
2	1257	1	0	0	1
3	909	1	0	0	1
4	712	1	1	1	1
5	582	1	1	1	1
6	400	1	0	0	1
7	219	1	1	1	1
HB09					
1	1277	1	1	1	1
2	1055	1	0	1	0
3	882	1	1	1	1
4	731	1	1	1	1
5	615	1	1	1	1
HB10					
1	5000	0	1	0	0
2	1217	1	1	0	0
3	919	0	0	1	1
4	747	1	0	1	1
5	595	1	1	0	1
6	462	1	0	1	1
HB11					
1	1593	1	1	1	1
2	1354	1	1	1	1
3	978	1	1	1	1
4	830	1	0	0	0
5	657	1	1	1	0
6	500	1	1	1	1
HB12					
1	1959	1	1	1	1
2	1304	1	0	1	1
HB13					
1	1278	1	1	1	1
2	997	1	1	1	1
3	756	1	1	0	0
4	605	1	0	0	0
5	557	1	1	1	0
6	421	1	1	0	0
Total	32	29	21	21	23

*bp= base pairs, present = 1 and absent = 0

three cultivar, multicut, Gemmiza1, Giza6, and Serw1. As well, the primer HB08 was observed to produce unique bands with the ecotype multicut for Serw1 cultivar at 1652 bp. Moreover, the primer HB08 was given 3 bands monomorphic, 4 bands polymorphic with 57.1% polymorphism (Table 3). Primer HB09 showed 5 bands with the two ecotypes. The bands were distributed in 4 monomorphic and 1 polymorphic with 20% polymorphism ratio (Table 3 and Figure 1). Primer HB10 was more variable than the other primers, which give 6 bands as a total polymorphic, 100% polymorphism, and one unique band with multicut ecotypes Egyptian clover Gemmiza1

Table 3. Primer name, total band, monomorphic, polymorphic, polymorphism ratio, unique bands and cultivar name.

Primer Name	Total band	Monomorphic Band	Polymorphic band	Polymorphism %	Unique band number	Cultivar name	MW (Bp)	Ecotype
HB08	7	3	4	57.1	1	Serw1	1652	Multicut
HB09	5	4	1	20.0				
HB10	6	0	6	100	1	Gemmiza1	5000	Multicut
HB11	6	4	2	33.3	4	Fahl	830	Monocut
HB12	2	1	1	50.0				
HB13	6	2	4	66.7	4	Fahl	605	Monocut
Total	32	14	18	56.3	10			

Table 4. Similarity indices among four Egyptian clover cultivars based on ISSR-PCR analysis.

Cultivars	Fahl	Gemmiza1	Giza6
Gemmiza1	0.82		
Giza6	0.80	0.78	
Serw1	0.81	0.70	0.82

at 5000 bp (Tables 2, 3 and Figure 1).

The cultivar Fahl (monocut ecotype) had four bands with ISSR primer HB10 which have molecular weight 1217, 747, 595 and 462 bp according to data in (Table 2 and Figure 1). On the other hand, the multicut ecotype cultivars which include three Egyptian clover cultivars Gemmiza1, Giza6 and Serw1 have different distribution regards to those four bands. The multicut ecotype cultivar Gemmiza1 were involved with monocut ecotype Fahl in 1217 and 595 bp as well as they disagrees in three bands (Table 2). The Fahl and multicut ecotype Giza6 were involved in two bands 747 and 462 bp. Furthermore, Fahl and Serw1 were involved in three bands 747, 595 and 462 bp but not 1217 bp (Table 2 and Figure 1).

Six bands appeared with primer HB11 out of which four bands were monomorphic, two bands polymorphic with 33.3% polymorphism ratio. Unique band at 830 bp was observed in Fahl cultivar only, and it was absent in other cultivars (Tables 2, 3 and Figure 1).

The primer HB12 produced two bands in both ecotypes at 1959 and 1304 pb except in Gemmiza1 cultivar band 1304 was absent. As well as, one band monomorphic, 1 band polymorphic with 50% polymorphism ratio and non-unique bands (Tables 2, 3 and Figure 1).

The primer HB13 amplified six bands with the two ecotypes with 2 bands monomorphic and 4 bands polymorphic with 66% polymorphism ratio. One unique band was found in Fahl monocut ecotype at 605 bp and was absent in multicut ecotype cultivars (Tables 2, 3 and Figure 1). All ecotypes were involved in bands number 1 and 2 that founded at 1278 and 997 bp (Tables 2, 3 and Figure 1). It was noted that the two ecotypes were evolved in band at 557 bp except serw1 which was absent in it (Tables 2, 3 and Figure 1).

It is worth mentioning that the Fahl had 29 present bands, 3 absent bands and two unique bands across the six primer. The multicut ecotypes were given different band pattern, Gemmiza1 (21 present, 11 absent), Giza6 (21 present and 11 absent) and Serw1 (23 present and 9 absent) (Tables 2, 3 and Figure 1). These differences stem from the location of the class environment where the temperature and humidity, product features carry the harsh conditions, as is the case in Serw1 and the genetic structure of both ecotypes (Table 2). Both ecotypes were also found to be varied from each other as indicated by various molecular markers (Zayed, 2013; Zayed et al., 2010). In addition, Soliman et al. (2010) found the monocut ecotype Fahl which was primitive than multicut ecotype.

The present results are in line with the results obtained by Tarrad and Zayed (2009) who studied the multicut ecotype cultivar and observed disagreement in the field performance, isozymes and RAPD-PCR reaction based on genetic material and performance of genetic materials within cuts.

Similarity and dissimilarity

Similarity indices among the four Egyptian clover cultivars based on ISSR analysis showed that the highest value appeared between Fahl and Gemmiza1 as well as Giza6 and Serw1 (82%) followed by Fahl and Serw1(81%). The lowest similarity value appeared between Gemmiza1 and Serw1 followed by Gemmiza1 and Giza6 (Table 4). These results are in agreement with that of Tarrad and Zayed (2009) who reported that the highest similarity indices was 0.85 between the two cultivars Sakha4 and Gemmiza1, while a lowest similarity index (70%) was observed between the Giza6 and Helaly.

Cluster analysis

The dendrogram shown in Figure 2 shows that the cultivars were divided into two main clusters; cluster 1 had Fahl and Gemmiza1 cultivars and cluster 2 had

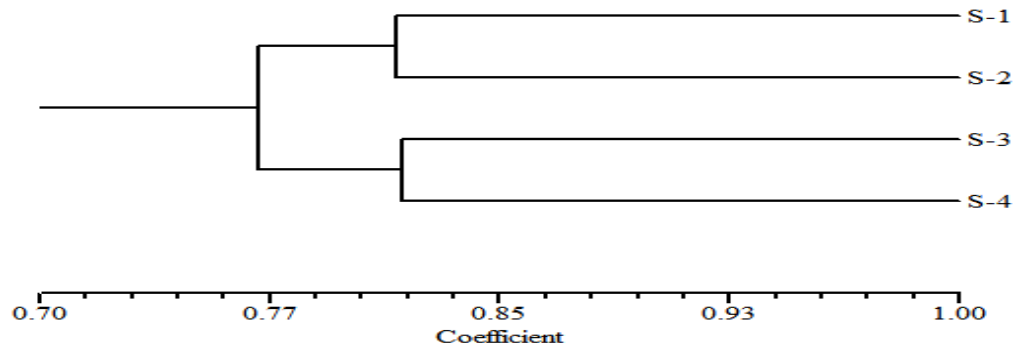

Figure 2. UPGMA clustering of Egyptian clover cultivars using the ISSR primers. S-1 = Fahl; S-2 = Gemmiza1; S-3 = Giza6; S-4 = Serw1.

Giza6 and Serw1 cultivars. These results are not in agreement with that of Tarrad and Zayed (2009) who reported the Gemmiza1 and Fahl had so far genetic distance and similarity indices equal to 50%.

The multicuts cultivars differed in the origins as follow: Gemmeiza1 developed through poly-crossing selections within collected landrace from Dusuok District, Kafr El-Sheikh Governorate, Egypt for high forage yield potential and prolonged re-growth period at Sakha Research Station. It has a vigorous agronomical traits, early flowering, higher tillering ability, and gives 4-6 cuts/ season (Middle Delta and Middle Egypt). The cultivar Serw1 developed through poly-crossing among 14 selected landraces characterized with high forage productivity under salt-affected soil at Serw1 Research Station. It is salt tolerant and gives 4-6 cuts/season (North Delta and salt affected soil). Moreover, the cultivar Giza6 developed through selection among farmer's seed lots. It has late flowering, heat tolerant good yielder and gives 4-6 cuts/season (Middle and Upper Egypt). On the other hand, Abd El-Naby et al. (2012) studied ISSR primers with Fahl and Sakha4 and its hybrids; analyses of ISSR gave a total number of 60 bands from five primers. Also, they found the number of polymorphic bands to be 44; while polymorphism percentage was 73.4%. Furthermore, Soliman et al. (2010) found the results may be important to distinguish the difference between monocut and multicut. They reported that Fahl is more primitive Miskawi. The selection of vigorous plants may be used to improve new cultivars with economic value and can increase forage production per unit area (Abd El-Naby et al., 2012; Abd El-Naby et al., 2009; Abo-Feteih et al., 2010). Moreover, the relationship study between two cultivars Fahl and Miskawi can be better performed using Cubic, Quadratic model (Zayed et al., 2010).

Allele frequency

A population is said to be in Hardy-Weinberg equilibrium when 5 conditions are met: no mutations, no gene flow (no immigration /emigration), large population size (no genetic drift), no selective forces and no non-random mating. The allele frequency had different values in both ecotypes (Table 5). The dominant allele was frequented in cultivars Fahl, Gemmiza1, Giza6 and Serw1 with values 0.8, 0.64, 0.68 and 0.74, respectively. These data mean the cultivar had differed in allele frequency.

Table 5. Allele frequency (p and q) in two cut ecotypes of Egyptian clover based on ISSR-PCR analysis.

Allele	Fahl	Gemmiza1	Giza6	Serw1
Dominant(p) present	0.80	0.64	0.68	0.74
Recessive (q)absent	0.20	0.36	0.32	0.26

An average polymorphic information content (PIC) value of 0.218 across all scored ISSR bands, as well as an average (Marker index) of 3.709 across all primers obtained with both ecotypes berseem clover were different than that of AFLP-based genetic diversity studies in various crops (Muminovic et al., 2004; Powell et al., 1996). Though both AFLP and RAPD are dominant markers, the easiness associated with RAPD analysis as well as high PIC and MI obtained with berseem clover justifies its use for fingerprinting and identification of cultivars for different groclimatic zones (Table 6).

Conclusion

The ISSR markers have been used in order to determine genetic variation and relationship between two Egyptian clover ecotypes. Six primers out of ten for ISSR-PCR technique succeeded and gave reliable amplicons for different types of Egyptian clover ecotypes. The results reveal polymorphisms level by ISSR primers. HB10 ISSR-primer was better than the rest of the ISSR primers in polymorphic 100%. The Fahl monocut ecotype had 29 present bands, 3 absent bands in 32 total bands; also

Table 6. Comparative analysis of banding patterns generated by ISSR for four berseem clover.

Primer Name	Polymorphism (%)	Range of fragment size (pb)	PIC*	MI**
HB08	57.1	1652.4-219	0.268	1.072
HB09	20.0	1276.7- 614.6	0.110	0.11
HB10	100.0	1216.9- 462.2	0.319	1.595
HB11	33.3	1593.2- 499.8	0.153	0.306
HB12	50.0	1959.3 -1303.7	0.183	0.183
HB13	66.7	1278 - 421.3	0.276	1.104
Mean	54.8		0.218	3.709

*PIC = Polymorphic information content; **MI = marker index.

Fahl had two unique bands. The multicut ecotype Gemmiza1, Giza6 and Serw1 were given different pattern of bands 21 present, 11 absent; 21 present and 11 absent and 23 present and 9 absent, respectively. The three unique bands appeared in two ecotypes. Fahl was given two bands HB11 and HB13; the Serw1 multicut cultivar had one unique bands with HB08. Similarity indices among the four Egyptian clover cultivars based on ISSR analysis was estimated and the highest value appeared between Fahl and Gemmiza1 as well as Giza6 and Serw1 followed by Fahl and Serw1, while the lowest similarity value was between Gemmiza1 and Serw1 followed by Gemmiza1 and Giza6.

Conflict of interests

The authors did not declare any conflict of interest.

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Incidence and antimicrobial susceptibility pattern of extended-spectrum-β-lactamase-producing *Escherichia coli* isolated from retail imported mackerel fish

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During the past few years, extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and other species of Enterobacteriaceae have become a matter of great concern in human and veterinary medicine. Several studies in recent years documented the prevalence and occurrence of ESBL-producing *Enterobacteriaceae* in food products such as meat, poultry and raw milk; therefore in this pilot study we examined imported raw frozen mackerel fish to determine the incidence of ESBL-producing *E. coli* from Eastern Province of Saudi Arabia. From January to March, 2012, 45 samples were purchased from various supermarkets of this region and examined for the presence of ESBL-producing *E. coli* using ChromID ESBL agar plates and further confirmed by PCR amplification. Out of 45 mackerel fish samples, 23 (51.1%) were found to be positive for ESBL-producing *E. coli* and yielded 60 isolates. The higher rate of resistance was found to be with ampicillin (100%), piperacillin (96.7%), cefotaxime (93.3%), ceftriaxone (93.3%), tetracycline (53.3%), nalidixic acid (40%) and trimethoprim (30%). The least rate of resistance was recorded among chloramphenicol (15%), ciprofloxacin (15%), noroxin (11.7%) and nitrofurantoin (5%). All the 60 isolates in this study were found susceptible to amikacin, aztreonam, cefepime, ertapenem, gentamicin and kanamycin. Further characterization by PCR revealed that 49 (82%) out of 60 isolates of ESBL-producing *E. coli* were confirmed to be bla_{CTX-M} type and were negative for bla_{TEM} and bla_{SHV} genes. This is the first study to demonstrate the occurrence of ESBL-producing *E. coli* in imported raw frozen mackerel fish in Saudi Arabia and the study result indicates that the mackerel fish might be the possible reservoir of bla_{CTX-M} gene and may contribute to the dissemination and transfer of these β-lactamase genes to humans through food chain. The high rate of occurrence of ESBL-producing *E. coli* in the mackerel fish indicates that there is an established reservoir of these bacteria in the mackerel fish. Further national wide studies are necessary to assess future trends in imported fish to Saudi Arabia.

Key words: Mackerel fish, *Escherichia coli*, extended-spectrum β-lactamase (ESBL), antimicrobial resistance, PCR.

INTRODUCTION

The increase and spread of extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* over the past decade has become a global problem (Bradford, 2001; Paterson and Bonomo, 2005; Babic et al., 2006;

Bush, 2008; Ozcakar et al., 2011). The ESBLs are plasmid-encoded enzymes that inactivate a large number of β-lactam antibiotics such as extended-spectrum, broad-spectrum cephalosporins and monobactams. These β-lactamases are commonly inhibited by β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam (Bush et al., 1995; Paterson and Bonomo, 2005). In several studies, across the globe reported alarming high rate of ESBL producing *Escherichia coli* not only in human infections (Paterson and Bonomo, 2005; Perez et al., 2007; Cantón et al., 2008; Poeta et al., 2008), but also in a wide range of food producing animals, (Ojer-Usoz et al., 2013), food products (Schmid et al., 2013) and environment (Mesa et al., 2006), showing that ESBL are not restricted to clinical settings alone. The microbiological safety of fish and other fishery products is an important public health concern throughout the world (FAO, 2010). A recent study from China has identified fish as a reservoir of ESBL producing *E. coli* (Jiang et al., 2012). Since the ESBLs are located on plasmids, many of them are derived from mutations in bla_{SHV} (Sulphydryl variable) and bla_{TEM} (Temoneira) genes determined by amino acid substitutions around the active site. Apart from SHV and TEM types, *E. coli* isolates may additionally produce CTX-M (cefotaximase-Munchen) enzymes. CTX-M β-lactamases are more active against cefotaxime and ceftriaxone than against ceftazidime, even though point mutations can increase their activity against ceftazidime as well (Manoharan et al., 2011). The CTX-M enzymes are being discovered throughout the world and are becoming the most prevalent beta-lactamases found in clinical isolates and now is considered the most prevalent ESBLs worldwide (Federico et al., 2007; Livermore et al., 2007; Bonnet, 2004; Canton and Coque, 2006). The CTX-M enzymes have been reported to be detected from different food products and food producing animals that were recognized as reservoirs for ESBL-producing *E. coli* (Carattoli, 2008; Geser et al., 2011; Egea et al., 2012). Recently, several studies have shown that these resistance genes entered and disseminated through the food chain via direct contact with humans and animals and could contribute to the spread of the these strains (Winokur et al., 2001; Oppegaard et al., 2001; Mesa et al., 2006; Egea et al., 2012).

There is no data available about ESBL-producing bacteria in food and aquaculture products and food of animal's origin in Saudi Arabia. Therefore, this study was conducted to estimate the incidence and to provide current baseline information on the antimicrobial resistance patterns and molecular characterization of ESBL-producing *E. coli* in imported mackerel fish purchased from several retails supermarkets in the Eastern Province of Saudi Arabia.

MATERIALS AND METHODS

Sampling and isolation of ESBL producing *E. coli*

A total of 45 imported mackerel fish samples with labeled information (the country of origin and the storage temperature) were purchased from different supermarkets in Eastern Province of Saudi Arabia. The purchased samples were collected and transported on ice bag to the Microbiology Research Laboratory, University of Dammam. Upon arrival, the samples were kept intact on ice and analyzed within 2 to 3 h of collection. The examination of the mackerel fish samples were carried out according to the Methods of Bacteriological Analytical Manual (BAM, 2011) and also other published protocol with modifications (Elhadi et al., 2004; Zhao et al., 2001). Briefly, 25 g of samples (fish gills, intestines parts and skin) were placed into a stomacher bag containing 225 ml of EC broth (Oxoid, UK) and homogenized using a stomacher (Seward Stomacher 400 Circulator, UK) for 2 min and incubated for 18 to 24 h at 35°C. After enrichment incubation, 0.1 ml was streaked on ESBL chromogenic agar and incubated overnight at 37°C, three to four pink to reddish colored colonies with distinct morphological were isolated and subjected to biochemical tests (indole positive and oxidase negative) and were further confirmed by using API 20E (bioMe´rieux, France).

Antibiotic susceptibility testing

Antimicrobial susceptibility was determined by the disk diffusion method on Muller-Hinton agar Plates (Oxoid, Baringstoke, Hampshire, United Kingdom) as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010). The isolates were tested against the following antibiotics (Baringstoke, Hampshire, United Kingdom) AK: Amikacin (30 µg); AP: Ampicillin (10 µg); AUG: augmentin (30 µg); ATM: aztreonam (30 µg); FEP: cefepime (30 µg); CTX: cefotaxime (30 µg); CAZ: ceftazidime (30 µg); CRO: ceftriaxone (30 µg); C: chloramphenicol (30 µg); CIP: ciprofloxacin (5 µg); ETP: ertapenem (10 µg); GM: gentamicin (10 µg); K: kanamycin (30 µg); NA: nalidixic acid (30 µg); NI: nitrofurantoin (300 µg); NOR: noroxin (10 µg); PRL: piperacillin (100 µg); T: tetracycline (30 µg); TN: tobramycin (10 µg); TM: trimethoprim (5 µg) and TS: Trimethoprim/sulfamethoxazole (25 µg). *E. coli* American Type Culture Collection (ATCC) 25922 was used as a reference strain for antimicrobial disk control.

Phenotypic confirmation of ESBL by E-test

E-test strips (Oxoid, UK) with concentration gradient of cefotaxime or ceftazidime at one end and cefotaxime or ceftazidime with Clavanic acid at the other end were accomplished according to the guidelines of the manufacturer for confirming ESBL production. ESBL production was determined by a ≥3 doubling dilutions decrease in the MIC of cefotaxime or ceftazidime in the presence of Clavanic acid. ESBL production was also recognized by the

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Abbreviations: ESBL, Extended-spectrum β-lactamase; **ND,** non-determinable; **BAM,** bacteriological analytical manual.

Table 1. Nucleotide sequences of PCR primers.

Gene	Primer sequence (5'-3')	Amplicon size (bp)
bla_{CTX-M}	F: CGCTGTTGTTAGGAAGTGTG R: GGCTGGGTGAAGTAAGTGAC	754
bla_{TEM}	F: TTTCGTGTCGCCCTTATTCC R: CGTTGTCAGAAGTAAGTTGG	403
bla_{SHV}	F: CGCCTGTGTATTATCTCCCT R: CGAGTAGTCCACCAGATCCT	293

Table 2. Percentage of antibiotic agents tested against *E. coli* strains isolated from imported mackerel fish (n=60).

Antimicrobial agents (µg)	Resistant, n (%)	Intermediate, n (%)	Susceptible, n (%)
Amikacin (30)	0	0	60 (100)
Ampicillin (10)	60 (100)	0	0
Augmentin (30)	1 (1.7)	0	59 (98.3)
Aztreonam (30)	0	50 (83.3)	10 (16.7)
Cefepime (30)	0	0	60 (100)
Cefotaxime (30)	56 (93.3)	4 (6.7)	0
Ceftazidime (30)	0	0	60 (100)
Ceftriaxone (30)	56 (93.3)	4 (6.7)	0
Chloramphenicol (30)	9 (15)	0	41 (68.3)
Ciprofloxacin (5)	9 (15)	0	51 (85)
Ertapenem (10)	0	0	60 (100)
Gentamicin (10)	0	0	60 (100)
Kanamycin (30)	0	0	60 100)
Nalidixic acid (30)	24 (40)	0	36 (60)
Nitrofurantoin (30)	3 (5)	0	57 (95)
Noroxin (10)	7 (11.7)	0	53 (88.3)
Piperacillin (10)	58 (96.7)	0	2 (3.3)
Tetracycline (30)	56 (53.3)	0	4 (6.7)
Tobramycin (10)	2 (3.3)	0	58 (96.7)
Trimethoprim (5)	18 (30)	0	42 (70)
Trimethoprim/sulfamethoxazole (25)	18 (30)	0	42 (70)

appearance of a phantom zone in the cefotaxime or ceftazidime strip. A non-determinable (ND) result was declared when the MICs were greater than the range of MICs of the respective E-test ESBL test strip.

Genotypic characterization of β-lactamases

All the isolates confirmed as ESBL producers were analyzed by using PCR amplification (Pitout et al., 1998; Poeta et al., 2008; Woodford et al., 2005). Genomic DNA was extracted by a standard heat lysis protocol and the same were used as the template. Amplification of TEM, SHV and CTX-M was performed with primer and cycling conditions presented in Table 1.

RESULTS AND DISCUSSION

Isolation of ESBL producing *E. coli*

A total of 60 isolates of ESBL-producing *E. coli* were retrieved from 45 frozen imported mackerel fish samples that were obtained from supermarkets in Eastern Province of Saudi Arabia. In the present study, ChromID ESBL agar plates were used according to manufacturer's guidelines. It was found to have excellent sensitivity and specificity in detecting ESBL producing *E. coli* resulting in reduction in time and amount of bench work.

Antibiotic susceptibility testing

Antibiotic susceptibility testing result are shown in Table 2. Among all the total 60 study isolates, the higher percentage of resistance were found with piperacillin (96.7%), cefotaxime (93.3%) and ceftriaxone (93.3%) while, lowest resistance found with Tobramycin (3.3%) and Nitrofurantoin (5%). All the isolates were found to be

Table 3. Antibiotic resistant patterns and number of ESBL producing gene of *E. coli* strains isolated from imported mackerel fish (n=60).

Antibiotic resistance pattern	Number of isolates	ESBL genes		
		bla_{CTX-M}	bla_{TEM}	bla_{SHV}
		n (%)	n (%)	n (%)
AP-PRL	1	1	0	0
AP-NI-AUG	1	0	0	0
AP-CRO-PRL	2	2	0	0
AP-T-CRO-CTX-PRL	23	20	0	0
AP-T-CRO-CTX-PRL-NA	15	13	0	0
AP-T-CRO-CTX-PRL-TS-TM	9	5	0	0
AP-T-C-NI-CTX-PRL-NA-TS-CIP-TN-TM	2	2	0	0
AP-T-C-CRO-CTX-PRL-NA-TS-CIP-NOR-TM	7	6	0	0
Total	60	49 (81.7)	0	0

resistant to ampicillin whereas, all isolates were found to be susceptible to amikacin, cefepime, ceftazidime, ertapenem, gentamicin, and kanamycin. Unexpectedly, one isolate was found resistant against augmentin. 83.3% isolates had intermediate susceptibility against Aztreonam raising serious concern. This decreasing Aztreonam susceptibility was in concordance to our recent unpublished study regarding antibiotic resistant *E. coli* in beef and shrimp isolated from the same region. Out of all the 60 strains of *E. coli*, 23 strains yielded the similar resistance pattern AP-T-CRO-CTX-PRL, 15 strains yielded resistance pattern AP-T-CRO-CTX-PRL-NA and 9 strains yielded resistance pattern AP-T-CRO-CTX-PRL-TS-TM, respectively (Table 3). All the study isolates were found to be resistant to two or more antibiotics. The least discovered resistance patterns were AP-PRL and AP-NI-AUG, found in only one isolate each. A wide range of antibiotic classes are being used extensively in aquaculture, including aminopenicillins, amphenicols, macrolides, aminoglycosides, nitrofurans, fluoroquinolones, sulphonamides and tetracyclines (Heuer et al., 2009). The main consequence of this is the selection of MDR strains in the gut flora of fishes. During consumption of such contaminated fish products, the MDR strains are transferred to human gut and enter the food cycle (Heuer et al., 2009).

A recent research data from Sweden has shown the acquisition of fecal carriage of CTX-M-type ESBL producing *E. coli* in travelers to different parts of the world from Sweden and with 32% acquisition rate after travelling to Asia (Tangden et al., 2010). Fecal carriage is believed to be the most important reservoir of ESBL-producing bacteria in the community (Kluytmans et al., 2013). Some published research data documented the contamination of food animals, retailed chicken meat and beef meat with the ESBL-producing *Enterobacteriaceae* (Smet et al., 2008; Machado et al., 2008; Doi et al., 2010; Overdevest et al., 2011; Kluytmans et al., 2013). Previous reports suggest that resistant *E. coli* strains are probably more likely to be transmitted from poultry to humans than are susceptible variants (Johnson et al., 2007). A recent survey comparing resistance rates among *E. coli* from humans vs. from poultry, pigs, and cattle in 11 European countries found strong, statistically significant correlations for various groups of antibiotics (Vieira et al., 2011).

Notably, for resistance to extended-spectrum cephalosporins, a significant correlation was found only between humans and poultry, implicating poultry as an important source for human-associated ESBL-producing *E. coli*. Infections with the antibiotic resistant bacteria and ESBL producing *Enterobacteriaceae* are associated with increased morbidity, mortality, and healthcare costs as a result of hospital acquired infections (Cosgrove, 2006; Tumbarello et al., 2010). ESBL-producing *Enterobacteriaceae* infections are increasingly frequent among community-dwelling patients without a history of hospitalization or antimicrobial use (Friedmann et al., 2009; Valverde et al., 2004; Dubois et al., 2010). Based on this finding imported mackerel fish is an important reservoir for ESBL-producing *E. coli*. Consumption of this contaminated fish with ESBL producing *E. coli* may lead to the transmission of genetic elements containing resistance genes to the human intestinal micro-biota. The abundant use of antimicrobial agents in a production animal poses threat to human health. This has recently spurred the US Food and Drug Administration to propose a ban on certain uses of cephalosporins for livestock (Schmidt, 2012).

Genotypic characterization of β-lactamases

All the 60 ESBL producing *E. coli* isolates were subjected to PCR analysis and 49 (82%) were positive for CTX-M and negative for TEM and SHV genes as shown in Table 3, Figures 1 and 2. The one isolate which was found resistant to Aztreonam was negative for CTX-M. The CTX-M production was highest among the *E. coli*

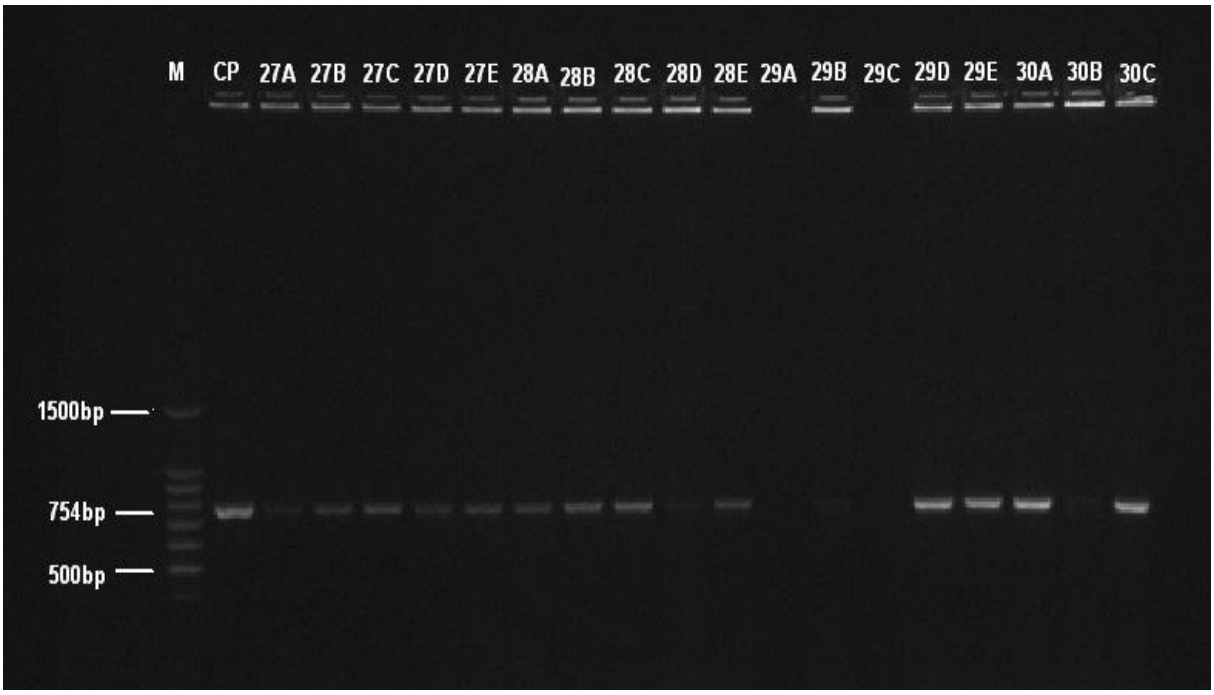

Figure 1. Representative Agarose gel electrophoresis of DNA amplification of selected *E. coli* isolates obtained with the PCR method. Lane M: Bench Top 100 bp DNA ladder (Promega, USA); Lane CP: Positive control. Lane 27A, 27B, 27C, 27D, 27E, 28A, 28B, 28C, 28E, 29D, 29E, 30A, 30C, CTX-M positive isolates. Lane 28D, 29A, 29B, 29C, and 30B, CTX-M negative isolates.

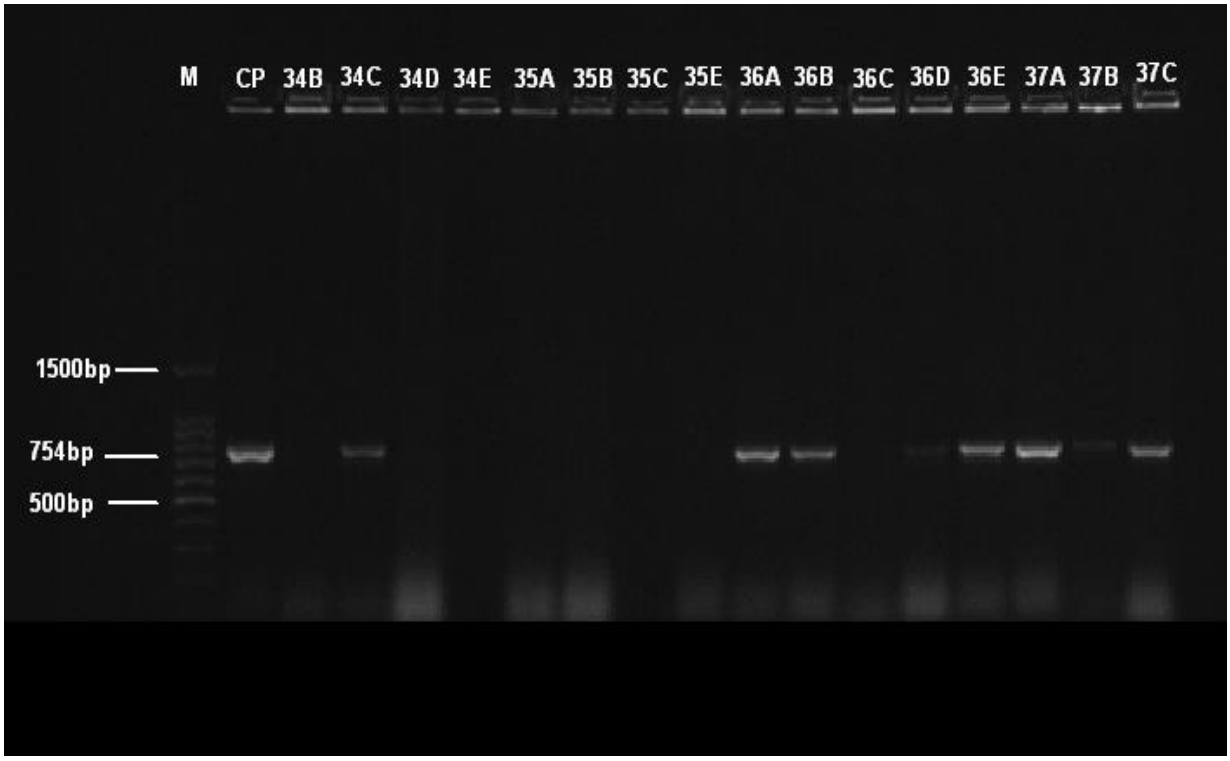

Figure 2. Representative Agarose gel electrophoresis of DNA amplification of selected *E. coli* isolates obtained with the PCR method. Lane M: Bench Top 100 bp DNA ladder (Promega, USA); Lane CP, Positive control. Lane 34C, 36A, 36B, 36E, 37A, and 37C: CTX-M positive isolates. Lane 34B, 34D, 34E, 35A, 35B, 35C, 35E, 36C, 36D, 37B: CTX-M negative isolates.

presenting the resistance pattern AP-T-CRO-CTX-PRL. TEM and SHV genes are often reported in ESBL-producing *Enterbacteriaceae* isolated from Poultry meat (Machado et al., 2008; Kola et al., 2012). The ESBL producing *E. coli* with CTX-M type and negative bla_{SHV} have been reported in wild birds in Germany (Guenther et al., 2010). Recent study from China demonstrated the presence of ESBL genes in farmed fish produced (Jiang et al., 2012). Infections due to ESBL-producing *E. coli* harboring ESBLs of the CTX-M classes have dramatically increased among human populations, particularly in the community setting (Livermore et al., 2007). There is very limited data available on the occurrence or prevalence of ESBL-producing *Enterobacteriaceae* in marine fish and in imported fish to Saudi Arabia. There is only one study to best of our knowledge, reported from China by Jiang et al. (2012) that described the prevalence of β- lactamase in *E. coli* isolated from farmed fish (Jiang et al., 2012). There is no data available on "pubmed" search (http://www.ncbi.nlm.nih.gov/) for Saudi Arabia and other Middle Eastern countries. As seen in our study, a predominance of CTX-M gene in *E. coli* isolated from food producing animals was reported in some European countries (Aarestrup et al., 2006; Girlich et al., 2007; Gonçalves et al., 2010).

Conclusion

The result of this study raises serious food safety concerns regarding the high prevalence of ESBL producing *E. coli* in the mackerel fish. This is the first study to report the high prevalence of ESBL-producing *E. coli* in the imported mackerel fish in Eastern Province of Saudi Arabia. The extensive prevalence of extended spectrum β-lactamase producing genes and high levels of co-resistance in *E. coli* detected in this study are of great concern and efforts should be made to monitor antibiotic resistance in aquaculture products, as this represents a major reservoir of antibiotic resistance. Larger studies should be undertaken in different geographical regions of the country to address these issues.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Screening of yeasts capable of producing cellulase-free xylanase

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Xylanases have largely been obtained from filamentous fungi and bacteria; few studies have investigated the production of this enzyme by yeasts. The aim of this study was to isolate yeasts from different sources, such as vegetables, cereal grains, fruits, and agro-industrial waste and to obtain yeasts capable of producing celulase-free xylanase. Samples were enriched using yeast malt broth, and yeasts were isolated on Wallerstein nutrient agar. In all, 119 yeast strains were isolated and evaluated in terms of their ability to degrade xylan, which was found in the medium by using agar degradation halos, the basis of this polysaccharide, and Congo red dye. Selected microorganisms were grown in complex medium and the enzymatic activities of endo-xylanase, β-xylosidase, carboxymetilcellulase, and filter paper cellulose were determined over 96 h of cultivation; the pH and biomass concentration were also evaluated. The yeast strain 18Y, which was isolated from chicory and later identified as *Cryptococcus laurentii*, showed the highest endo-xylanase activity (2.7 U.mL^{-1}). This strain had the ability to produce xylanase with low levels of cellulase production (both CMCase [0.11 U.mL^{-1}] and FPase [0.10 U.mL^{-1}]). This result gives this strain great biotechnological potential since this enzyme can be used for industrial pulp and paper bleaching.

Key words: *Cryptococcus laurentii*, endo-xylanase, xylan.

INTRODUCTION

Xylan, the major component of the hemicellulose complex, is a heterogeneous polysaccharide found in the plant cell wall, formed by xylopyranosyl residues linked by β-1,4-glycosidic bonds (Flores, 1997). An enzymatic complex is responsible for the total hydrolysis of xylan, but its main enzymes are endo-1,4-β-xylanase and β- xylosidase (Polizeli, 2005). These enzymes are responsible for the hydrolysis of the main chain: the former attacks the main internal chain of xylosidic linkages and the latter yields xylosyl residues by endwise attack of xylooligosaccharides (Subramaniyan and Prema, 2002).

Endo-1,4-β-xylanase and β-xylosidase are divided into two categories: one is associated with polysaccharidases (for example, cellulases) and the other is cellulase-free (Biely, 1985). The former is used for the production of hydrolysates in food and beverage processing (Sreenath,

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1996), in animal feed, to reduce viscosity and improve the digestibility of nutrients (Ahmad et al., 2013; Viana, 2011; Mathlouthi, 2003) and production of xylitol used as a natural sweetener (Parajó et al., 1998; Usama et al., 2013). Cellulase-free xylanase is used for paper and vegetable fiber bleaching (Damiano, 2003; Bocchini et al., 2003; Techapun et. al., 2003; Shangi, 2009).

Cellulase-free xylanase is used mainly in the pulp and paper industries for pretreating pulp prior to bleaching to minimize the use of chlorine, the conventional bleaching agent. This application has great potential as an environmentally safe method (Subramaniyan, Prema, 2000).

These enzymes are produced by molds, bacteria, yeasts, marine algae, among other organisms, but their main commercial sources are filamentous fungi (Polizeli et al., 2005). Xylanases from filamentous fungi are generally associated with the production of cellulases (Steiner, 1987), which may not be desirable, depending on the application of the enzyme.

Xilanolitic systems have largely been obtained from filamentous fungi (Adesina, 2013; Rao, 2002, Cacais, 2001) and bacteria (Rajagopalan et al. 2013; Chavez et al., 2006); there are few studies on the production of this enzyme by yeasts (Lara et al., 2014; Lopes, 2011; Pe9trescu, 2000). To the best of our knowledge, there have been no studies on the production of hydrolytic cellulases (CMCase and FPase) and β-xylosidase associated with the production of xylanases by yeasts; the only related studies have been conducted on filamentous fungi (Malabadi, 2007; Petrescu, 2000; Gottschalk, 2013).

It is important to isolate yeast strains that produce the xylanase enzyme to develop new process for synthesizing products of biotechnological interest. Therefore, the aim was to isolate and select yeasts from food and residues capable of producing cellulase-free xylanase enzymes.

MATERIALS AND METHODS

Sample collection

Samples were obtained from different cities in southern Rio Grande do Sul: Pelotas (31º63´S/52º33´W), Rio Grande (31º21´S/52º38´W), São Lourenço (31º25´S/52º13´W), Quinta (32º08´S/52º26´W), and Povo Novo (31º93´S/52º30´W). The samples collected were vegetables (chayote, chicory, radicci, tomato), grains (soybeans, corn, rice), fruits (banana, jelly palm, grape), and agro-industrial wastes (rice husk, corn cobs, soybean hulls). Samples (10 g) were collected, stored in sterile plastic bags and transported under refrigeration to the laboratory within 24 h. For the isolation of yeasts, parts of the fruits and vegetables (stems, leaves, roots) were evaluated separately.

Yeast isolation

For enrichment, about 2.5 g of each sample was inoculated into 25 mL yeast malt broth composed of 3 $g.L^{-1}$ yeast extract; 3 $g.L^{-1}$ malt extract; 5 $g.L^{-1}$ peptone; and 10 $g.L^{-1}$ glucose (pH 6.0); the samples were then incubated at 25ºC for 72 h at 150 rpm (Maugeri, 2007).

Cultures were streaked onto Petri dishes with Wallerstein nutrient agar composed of 4 $g.L^{-1}$ yeast extract; 5 $g.L^{-1}$ bactocasitone; 50 $g.L^{-1}$ dextrose; 550 $mg.L^{-1}$ KH_2PO_4; 125 $mg.L^{-1}$ KCl; 250 $mg.L^{-1}$ $MgSO_4 \cdot 7H_2O$; 2.5 $mg.L^{-1}$ $FeCl_3$; 2.5 $mg.L^{-1}$ $MnSO_4$; 22 $mg.L^{-1}$ Bromocresol green, and 20 $g.L^{-1}$ agar at pH 5.5. Cultures were grown at 25°C for 72 h. Colonies were transferred and maintained in GYMP agar (20 $g.L^{-1}$ glucose, 5 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ malt extract, 2 $g.L^{-1}$ KH_2PO_4, and 20 $g.L^{-1}$ agar at pH 5.5) at 4°C in a slant tube (Maugeri, 2007). HCl was used to adjust the pH.

Screening yeast for xylanase activity

For the selection of yeasts capable of producing xylan-degrading enzymes, all isolated yeasts were tested using two different methods. In Method 1 (Whitaker, 2002), only a medium containing xylan (2 $g.L^{-1}$) and agar (17 $g.L^{-1}$) was used. In Method 2 (Lopes, 2011), the following medium was used: 10 $g.L^{-1}$ Beechwood xylan (Sigma); 0.6 $g.L^{-1}$ yeast extract; 7.0 $g.L^{-1}$ KH_2PO_4; 2.0 $g.L^{-1}$ K_2HPO_4; 0.1 $g.L^{-1}$ $MgSO_4.7H_2O$; 1.0 $g.L^{-1}$ $(NH_4)_2SO_4$, and 15 $g.L^{-1}$ agar; HCl was used to adjust the pH to 5.0. From the yeasts in GYMP agar, the inoculum was transferred to xylan agar plates, by picking one by one using a toothpick and incubated at 30°C for 72 h. The clearance zones produced around the yeast colonies in the medium were visible when Congo red was used. They were discolored by sodium chloride at different concentrations.

The diameters of the zones of clearance and colonies were measured in millimeters and the enzymatic index (EI) was calculated by Equation 1:

$$EI = \frac{diameter\ hydrolysis\ zone}{diameter\ colony}. \quad (1)$$

Strains with an EI above 1.0 were considered to be potential producers of xylanase in Method 1 (Whitaker, 2002), whereas strains with an EI above 2.5 were considered to be potential producers in Method 2 (Lopes, 2011).

Production of xylanase in liquid medium

Only the yeast strains considered to be potential producers of xylanase (which met the criteria described above) were pre-grown in liquid medium. The inoculum was grown in 150 mL medium, as follows: 10.0 $g.L^{-1}$ Beechwood xylan; 3.0 $g.L^{-1}$ yeast extract; 7.0 $g.L^{-1}$ KH_2PO_4; 2.0 $g.L^{-1}$ K_2HPO_4; 0.1 $g.L^{-1}MgSO_4.7H_2O$; 1.0 $g.L^{-1}$ $(NH_4)SO_4$ and 5.0 $g.L^{-1}$ peptone; the samples were grown at pH 6.0 (adjusted using HCl), 30ºC, and 150 rpm for 24 h (Lopes, 2011). The cultivation was carried out in 500 mL Erlenmeyer flasks containing 150 mL of the same medium and was inoculated with cellular suspension (10^8 cells.mL^{-1}) obtained from a 24 h inoculum, incubated in a rotary shaker at 30°C and 150 rpm. The samples, which had been collected over 96 h of fermentation (samples were taken every 12 h until peak activity was observed, after which they were immediately withdrawn every 24 h), were centrifuged at 6,000 × *g* at 4°C for 10 min. The cell-free supernatant was used to determine pH and the activities of enzymes (endo-xylanase, cellulase, and β-xylosidase). The precipitated cells were then used for biomass determination; the tests and analytical measurements

were performed in triplicate.

Analytical methods

Enzyme assay

Carboxymethylcellulase activity was assayed using a reaction medium containing 0.5 mL substrate (2% [w/v] solution of carboxymethylcellulose) and 0.5 mL crude enzymatic extract and incubated for 30 min at 50ºC. Reducing sugars were assayed as glucose using the DNS method (Miller, 1959). Filter paper activity was assayed using a reaction medium containing 0.5 mL crude enzymatic extract, 1.0 mL citrate sodium buffer (pH 4.8), and 50 mg filter paper incubated for 60 min at 50ºC (Ghose, 1989). One international unit of filter paper activity was considered as the amount of enzyme that forms 1 µmol glucose (reducing sugar as glucose) per min during the hydrolysis reaction. One enzyme activity unit (U) of Carboxymethylcellulase was defined as the amount of enzyme required to produce 1 µmol of glucose per min under the assay conditions..

For all enzymatic activities, blanks were performed with enzymes (buffer + enzyme) and substrates (buffer + substrate). All tests of enzymatic activities were performed in triplicate.

Biomass determination

The biomass concentration was estimated by measuring absorbancy in a spectrophotometer at 620 nm and relating the readings to biomass dry weight with a calibration curve (Rech et al., 1999).

Kinetic parameter determination

Kinetic parameters were determined for each growth curve: maximum biomass concentration (X_{max}, $g.L^{-1}$), maximum specific growth rate (μ_{max}, $1.h^{-1}$), and enzymatic productivity (P, $U.mL^{-1}.h^{-1}$) of each selected yeast grown in liquid medium, as described in "production of xylanase in liquid medium" section of this work.

The productivity (P) was calculated when the time of maximum activity was reached, and the maximum specific growth rate (μ_{max}) was calculated using Equation 2 by exponential regression of the logarithmic growth curve (Bailey and Ollis, 1986). The enzymatic productivity was calculated using Equation 3, according to Schmidell et al. (2001).

$$\mu = \frac{1}{x}\frac{dx}{dt} \quad (2)$$

$$P = \frac{A - A_0}{t - t_0} \quad (3)$$

Where, µ is the specific growth rate (h^{-1}), X is the biomass concentration ($g.L^{-1}$), A_o is initial enzymatic production, t is cultivation time at which maximum enzymatic activity occurs and t_o is the start point of cultivation.

Yeast identification

Only the yeast strain with the highest xylanase activity was identified. Identification was based on the sequencing of the D1/D2 domains of the large subunit of the rRNA gene. The D1/D2 domains were amplified by PCR, directly from whole cells as previously described (Lachance et al., 1999). Sequencing was performed on an ABI 3130 automated DNA gene analyzer according to the manufacturer's instructions. The sequences were assembled, edited, and aligned using the program MEGA6 (Tamura et al., 2013). The sequences obtained were compared to those included in the GenBank database using the Basic Local Alignment Search Tool (BLAST at http:// www.ncbi.nlm.nih.gov).

Statistical analysis

The results were evaluated statistically through Variance Analysis and Tukey test at 95% confidence level ($p < 0.05$), using Statistica 5.0 software.

RESULTS AND DISCUSSION

In all, 119 yeasts strains were isolated from different types of samples to obtain microorganisms capable of producing xylanolytic enzymes. Strains that were capable of degrading xylan present in the medium and that met the criteria of both methods were chosen. Results were expressed in terms of rings of hydrolysis, revealed by the addition of Congo red dye. Among the strains tested, 23 were able to hydrolyze xylan, but only 7 of those (6% of all microorganisms) met the criteria of both methods and thus were selected (Table 1). These results were considered promising since they are similar to those of previous studies. For example, Rao et al. (2008) obtained 374 yeasts from tree bark and decaying fruits that were evaluated for the ability to ferment xylose, and found that 27 isolates (7.2%) converted it into ethanol. Similarly, Lopes et al. (2011) evaluated 349 yeasts in Petri plates, and found that 9 (2.6 %) showed an enzymatic index above 2.5.

The enzymatic index, a semi-quantitative parameter applied to evaluate the ability of yeast strains to produce enzymes in solid medium, has been used by several authors (Ribeiro et al., 2014; Adesina, 2013; Florencio, 2012; Nagar, 2012; Samanta et al., 2011; Tallapragada, 2011), who have considered it an efficient method for screening for microorganisms. The next selection step was performed according to the capacity of yeast strains to produce the xylanolytic enzyme in liquid medium with xylan. Based on the activity of endo-xylanases in the seven yeasts under study (Figure 1a), yeast 18Y showed the highest xylanolytic activity (2.7 $U.mL^{-1}$), followed by yeast 34Y (2.1 $U.mL^{-1}$), after 36 h of cultivation. Yeast 19Y (0.5 $U.mL^{-1}$) had the lowest ability to produce the enzyme endo-xylanase. As shown in Figure 1, yeast 19Y had the highest peak activity in the fastest culture time (12 h), followed by yeast 13Y, which needed 24 h to reach its maximum enzymatic activity. Yeast 40Y needed most time (48 h) to reach its maximum compared to the other yeasts, which had high production up to 36 h of cultivation followed by a significant decrease after 48 h of cultivation. The only exception was yeast 60Y since it did not show any meaningful decrease in its activity. β-Xylosidases are glycoside hydrolases, which catalyze the

Table 1. Enzymatic index of yeasts isolated from different samples

Strain	Samples	Enzymatic index (>1.0) (Method 1)	Enzymatic index (>2.5) (Method 2)
13Y	Tomato	6.0	4.0
18Y	Chicory	3.0	3.0
19Y	Chicory	2.3	2.5
34Y	Tomato	3.0	3.5
40Y	Corn Seed	2.0	2.7
53Y	Tomato	2.5	3.0
60Y	Chicory	4.0	2.5

release of xylose units from xylo-oligosaccharides derived from the degradation of xylan; thus, their presence may prove to be unsatisfactory when the aim is to obtain xylo-oligosaccharides (Subramaniyan and Prema, 2000). Therefore, the low levels of β-xylosidases produced by yeasts 18Y (0.003 $U.mL^{-1}$) and 34Y (0.0006 $U.mL^{-1}$) can be considered a positive factor. Low activities similar to the ones found in this study (Figure 1b) were also found by Biely (1980), who reported β-xylosidase levels below 0.1 $U.mL^{-1}$ produced by *Cryptococcus albidus* CCY 1741 in medium containing Beechwood xylan, and Bastawde (1994), who reported low levels of β-xylosidase by a yeast isolated from decaying wood (NCIM 3574). However, when cultivation is conducted with filamentous fungi, the levels of β-xylosidase tend to be higher, as reported by Lemos (2000). This author described β-xylosidase activities of 1.3 $U.mL^{-1}$ when cultivating *Aspergillus awamori* in medium with sugar cane bagasse.

In the present study, the production of cellulases (CMCase and FPase) was extremely low since all yeast activities were lower than 0.11 $U.mL^{-1}$ (Figure 1c and d). Therefore, xylanases produced by the yeasts under study could be applied to the textile (degumming of natural fibers), pulp, and paper industries. The values of cellulase found in this study agreed with the results of free-cellulase xylanase (0.02 $U.mL^{-1}$ for CMCase and FPase) from *Thermomyces lanuginosus* reported by Mendoza (2006). Boddireddy (2011) obtained free-cellulase xylanase from different isolated fungi with CMCase activities of 0.1 to 0.6 $U.mL^{-1}$ in submerged cultures. Alves-Prado (2010) cultivated *Lysinibacillus sp*. strain P5B1 in submerged cultures with xylan to produce endo-xylanase and reported a CMCase activity of 0.58 $U.mL^{-1}$. Therefore, considering these previous results, the findings obtained by the present study are promising.

The pH of the medium ranged from 6.0 to 7.2 (Figure 2a) during the cultivation of yeasts aimed at the production of xylanase. The only exception was yeast Yeasts 18Y and 34Y reached maximum enzyme 60Y: its initial pH was 6.2 but it reached 7.6 after 96 h. production within the first 36 h of culture at pH 7.1 and 6.7, respectively. Lopes (2011) isolated environmental yeasts and produced xylanase in medium with xylan. They observed that yeasts LEB-AY 10 and LEB-AAD5 reached their maximum enzymatic activity when the pH was around 6.5.

Regarding the production of biomass (Figure 2b), yeast 34Y stood out in terms of cell growth since it reached 12.27 $g.L^{-1}$. Yeast 18Y showed a maximum peak of endo-xylanase activity of 2.7 $U.mL^{-1}$, coinciding with the maximum increase in biomass production (5.75 $g.L^{-1}$), which occurred after 36 h.

The X_{max} found in the yeasts under study (Table 2) ranged from 2.54 $g.L^{-1}$ (yeast 53Y) to 12.27 $g.L^{-1}$ (yeast 34Y), whereas the productivity ranged from 0.015 $g.L^{-1}.h^{-1}$ (yeast 53Y) to 0.07 $g.L^{-1}.h^{-1}$ (yeast 18Y). The highest maximum specific growth rate (0.23 h^{-1}) was obtained by yeast strain 53Y while yeast 34Y had the lowest one (0.05 h^{-1}).

Yeast 18Y, which showed the highest endo-xylanase activity, was submitted to sequencing of the D1/D2 domains of the large subunit of the rRNA gene, and identified as *Cryptococcus laurentti*. Studies using micro-organisms for xylanase production are mainly performed using filamentous fungi (Guimarães, 2006) and bacteria (Vieira, 2007). The yeasts described in the literature as producing endo-xylanase include *Trichosporon* (Stevens, 1977), *Pichia stipitis* (Lee et al., 1986), *Aureobasidium pullulans* (Leathers et al., 1986), *Cryptococcus albidus* (Biely, 1980), and *Cryptococcus flavus* (Parachin et al., 2009; Yasui, 1984). Lopes (2011) isolated two strains of *Cryptococcus* that produced endo-xylanase with activities of 0.67 and 0.73 $U.mL^{-1}$. Morais et al. (2013) isolated yeasts from decaying wood and tested their ability to ferment xylose and produce endo-xylanase; the main yeasts were *Cryptococcus laurentti*, *Cr. humicola*, *Cr. flavences*, and *Cr. podzolicus*. Lara et al. (2014) isolated *C. laurentti* UFMG-HB-48, which
produced extracellular xylanase with an activity of nearly 1.3 $U.mL^{-1}$.

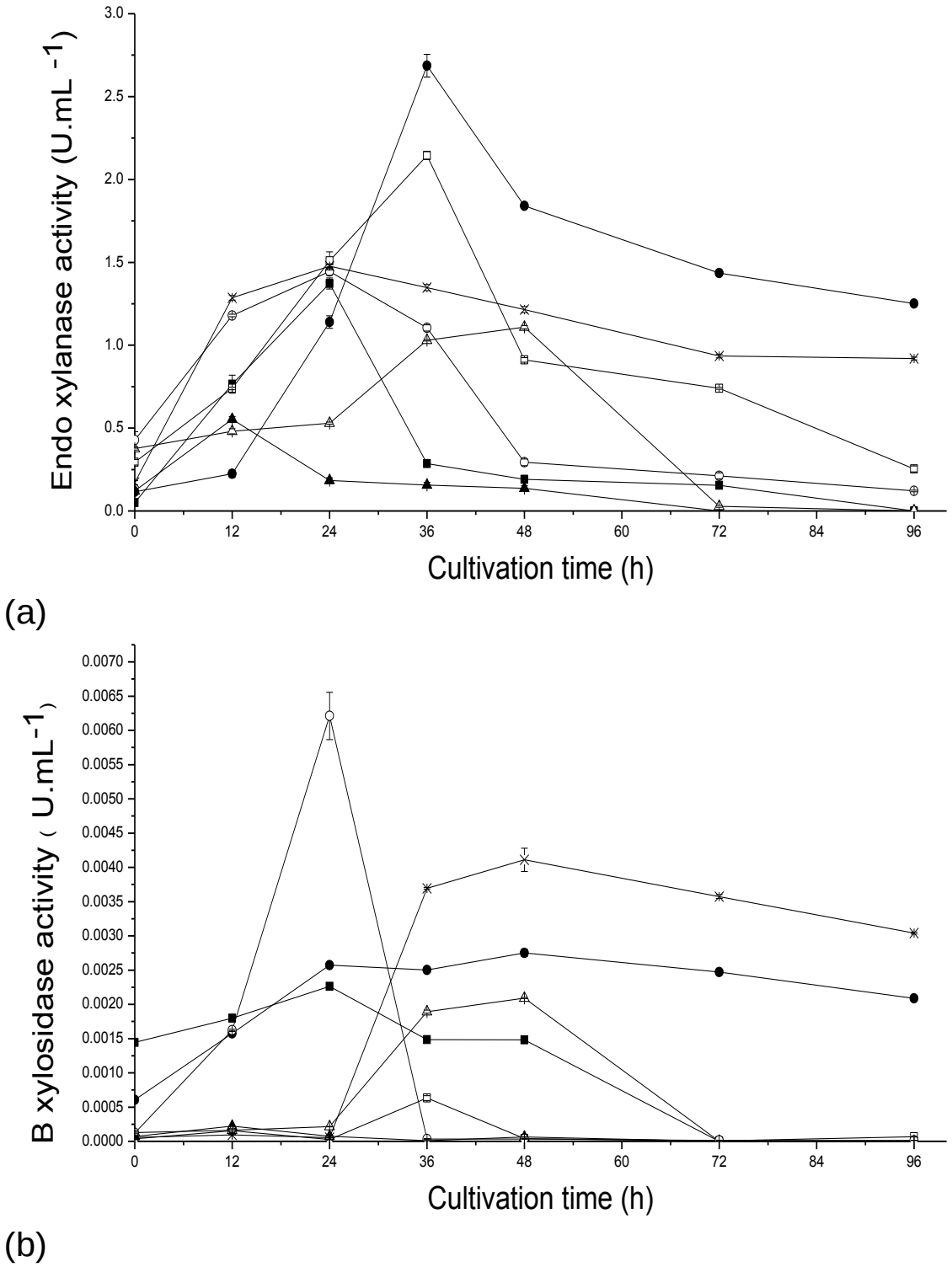
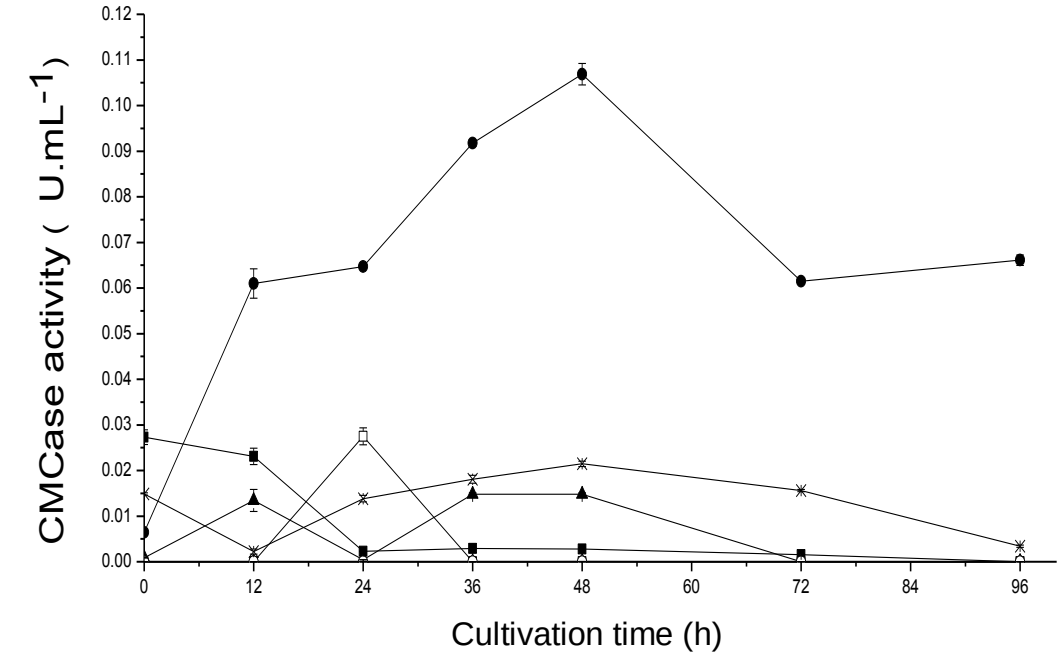
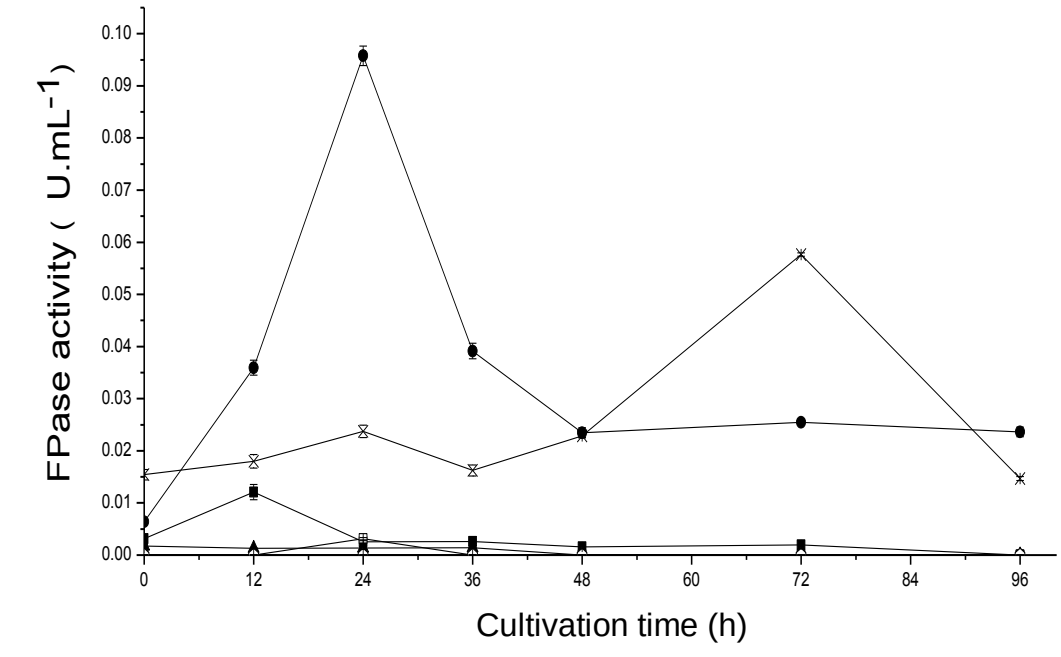


Figure 1. Activities of endo-xylanase (a), β-xylosidase (b), CMCase (c), and FPase (d) in the isolated yeasts in medium with xylan as its carbon source. (■) Yeast 13Y; (●) Yeast 18Y; (▲) Yeast 19Y; (□) Yeast 34Y; (Δ) Yeast 40Y; (o) Yeast 53Y; and (×) Yeast 60Y.

The enzymatic activities obtained in the present study are relevant not only because xylanase was produce d from yeasts but also because the activities that were observed were found under conditions that had not been



(c)

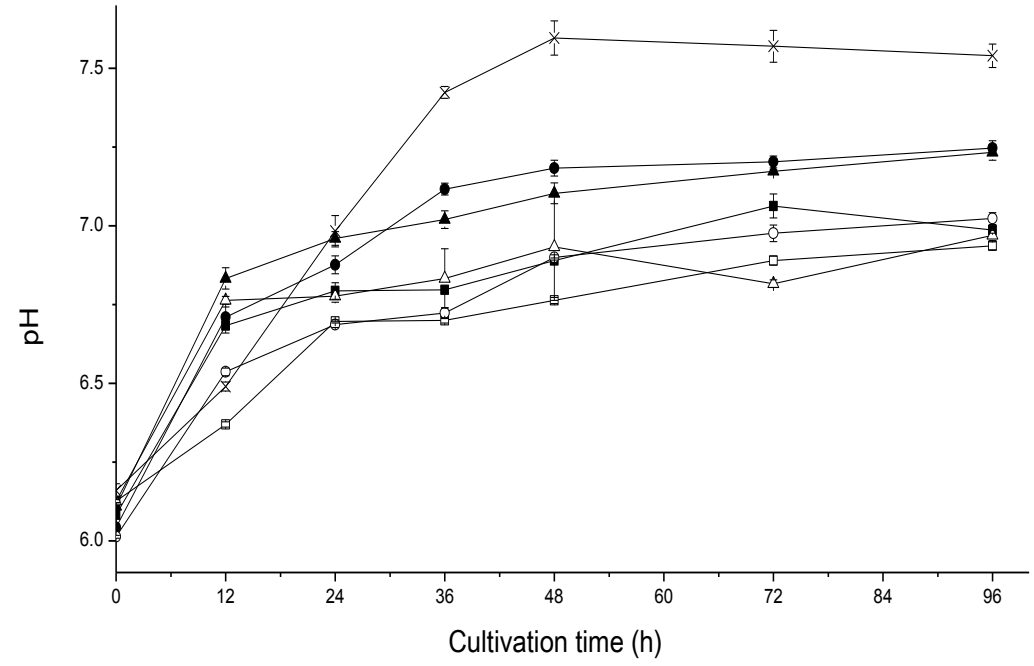


(d)

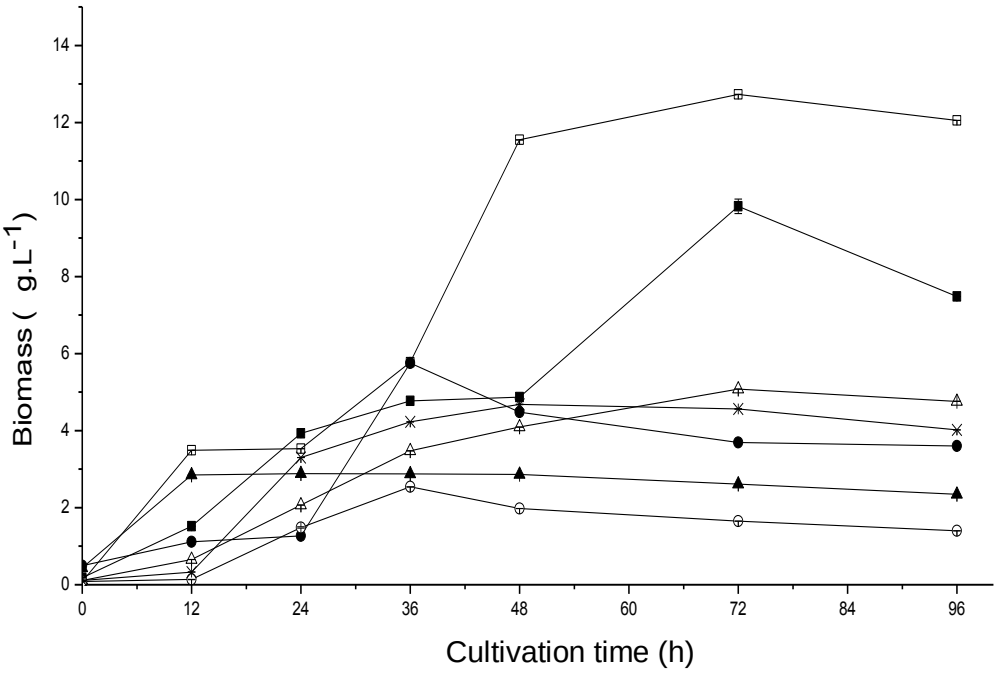
Figure 1. Contd.

optimized in terms of pH, temperature, stirring speed, the addition of ions, and so forth. Thus, their activities could be higher than observed.

The results also indicate that the samples used were suitable for the isolation of yeasts capable of producing xylan-degrading enzymes. Out of 119 isolated yeast strains, *C. laurentti* 18Y (isolated from chicory) showed the highest production of endo-xylanase (2.7 $U.mL^{-1}$); the averages of all the yeasts were evaluated statistically throughout the cultivation, as well as the maximum



(a)



(b)

Figure 2. The pH (a) and biomass (b) of the isolated yeasts in medium with xylan as its carbon source over 96 h, where: (■) Yeast 13Y; (●) Yeast 18Y; (▲) Yeast 19Y; (□) Yeast 34Y; (Δ) Yeast 40Y; (o) Yeast 53Y; and (×) Yeast 60Y.

temperatures were compared, where the yeast was statistically superior 18Y of other yeasts. This yeast species had the ability to produce endo-xylanase that was free of cellulase (CMCase and FPase) and showed low levels of β-xylosidade.

The potential industrial application of such cellulase-free xylanase, especially from yeast strains that show activity at a neutral pH, will be attractive to the paper and

Table 2. Kinetic parameters of isolated yeasts ± standard deviation.

Yeast	X_{max} ($g.L^{-1}$)	μ_{max} (h^{-1})	Productivity ($U.mL^{-1}.h^{-1}$)
13Y	9.82 ± 0.180	0.13 ± 0.002	0.05 ± 0.0021
18Y	5.75 ± 0.001	0.13 ± 0.001	0.07 ± 0.0016
34Y	12.27 ± 0.001	0.05 ± 0.001	0.05 ± 0.001
40Y	5.08 ± 0.001	0.11 ± 0.001	0.01 ± 0.001
53Y	2.54 ± 0.001	0.23 ± 0.007	0.05 ± 0.001
60Y	4.68 ± 0.005	0.13 ± 0.001	0.05 ± 0.001
19Y	2.88 ± 0.001	0.16 ± 0.001	0.03 ± 0.001

pulp industries to improve the quality of paper pulp as well as to minimize environmental pollution, which occurs due to the use of hazardous chemicals by these industries. In addition, these xylanases could also be used in agriculture; in the production of human food, cattle feed, and pet food; and in the production of xylo-oligosaccharides for pharmaceutical purposes.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Recovery of active pathogenesis-related enzymes from the apoplast of *Musa acuminata* infected by *Mycosphaerella fijiensis*

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The fungus *Mycosphaerella fijiensis* causes black Sigatoka (BS) disease, a major pathogen in the banana industry worldwide. Numerous molecular and biochemical studies have been done for the *M. fijiensis*, *Musa acuminata* interaction, but this is the first study describing the zymographic behavior of β-1,3-glucanase, chitinase and protease in the apoplast and symplast of healthy, BS-infected but asymptomatic and BS-diseased banana leaves. In BS-infected tissues, β-1,3-glucanase enzymatic activity was associated with two polypeptides with retention index (R_i) values of 0.43 and 0.56. These were more notable in the apoplast than in the symplast. Chitinase activity in BS-infected tissue in both the apoplast and symplast was mainly associated with a single polypeptide (R_i = 0.89). Both β-1,3-glucanase and chitinase activities were apparently more intense in BS-infected leaves than in healthy leaves. Protease activity was associated with two polypeptides (R_i = 0.04 and 0.14). In both the apoplast and symplast, the R_i 0.04 polypeptide increased in intensity with disease progression, whereas R_i 0.14 polypeptide intensity decreased. Overall protease activity intensity was higher in the symplast. Maximum symplast contamination of the apoplast was 2% as estimated by glucose 6-phosphate dehydrogenase activity, a biochemical marker for symplast. Accumulation of pathogenesis-related enzymatic activities in the apoplast of *M. acuminata* leaf tissue was caused by host-controlled enzyme downloading in response to *M. fijiensis* infection. Clear differences were identified in the electrophoretic profiles of healthy and diseased banana plants. The results further support a putative role of these enzymes in the extracellular defense repertoire of banana and, more importantly, suggest that *M. fijiensis* possesses a mechanism for suppression and delay of defense response in *M. acuminata*.

Key words: Black Sigatoka, glucose 6-phosphate dehydrogenase, pathogenesis-related (PR) proteins, polyacrylamide gel electrophoresis (PAGE), retention index (Ri), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

The apoplast is a continuous space surrounding plant cells, and is comprised of the cell wall matrix and intercellular spaces. Constituting 5 to 10% of plant mass (Gau et al., 2004), it plays a major role in a wide range of physiological processes. Its chemical composition is mostly water, ions and proteins, and it may be dependent on tissue distribution (Gau et al., 2004; Sattelmacher, 2001). The apoplast functions in cell signaling and plant

defense suggest it plays a key role during plant-pathogen interactions (Gau et al., 2004; Joosten and De Wit, 1989). During defense responses, the apoplast may contain over 200 proteins (Robertson et al., 1997). These are exported from the cell interior, mostly via the endoplasmic reticulum and the plasma membrane, but also via the plasma membrane through different, still incompletely understood routes (Gau et al., 2004; Hoson, 1998;=Floerl et al., 2008; Shabab et al., 2008). When bacteria and fungi attempt to gain access to the cell apoplast through the stomata or cuticle, the plant cell must respond to this challenge to protect itself. This can be accomplished via secretion of proteins that exert protective functions (Floerl et al., 2008), including a vital group known as pathogenesis-related (PR) proteins (van Loon and Kammen, 1970; van Loon and Strien, 1999; Blein et al., 2002; Brito-Argáez et al., 2010). Several research groups have used different approaches to analyze apoplast content during plant-pathogen interaction (Floerl et al., 2008; Shabab et al., 2008). Proteomics is increasingly popular, but has serious disadvantages in recovery of proteins with associated enzymatic or structural functions (Haslam et al., 2003; Boudart et al., 2005; Zhou et al., 2011). Extraction and characterization of the apoplastic proteins involved in pathogenesis or plant defense is an important strategy for study of specific pathosystems. This method is known to be feasible in diverse plant-pathogen interactions for example *Cladosporium fulvum-Lycopersicon esculentum* (Joosten and De Wit, 1989); *Leptosphaeria maculans-Brassica napus* (Brownfield and Howlett, 2001); non-pathogenic bacteria-*Malus domestica* (Kürkcüoglu et al., 2004); *Verticillium longisporum-Brassica napus* var. napus (Hoson, 1998); and *Septoria tritici-Triticum aestivum* (Shetty et al., 2009). Although, they are biologically significant, research into apoplastic proteins is hampered by their low abundance relative to overall intracellular protein concentration (Haslam et al., 2003; Mendoza-Rodríguez et al., 2006).

The fungus *Mycosphaerella fijiensis* is the most significant threat to banana and plantain production worldwide. This pathogen causes the disease known as black Sigatoka (BS) or black leaf streak (Churchill, 2011). It affects leaf tissues, thus reducing photosynthetic area, and leads to premature fruit ripening and important production losses (De Bellaire et al., 2010). It is hemibiotrophic, with an initial biotrophic mode of nutrition, a long asymptomatic period, and finally necrotrophy, which produces visible symptoms (hence the term black Sigatoka). During biotrophy, hyphae grow through the mesophyll layers, colonizing intercellular space (De Bellaire et al., 2010; Churchill, 2011). During this stage, the fungus is believed not to cause any host damage because it uses the available host resources to support its own growth. When host resources are insufficient to support continued hyphae growth, the fungus transitions to the necrotrophic stage and begins to secreting effectors (that is, secondary metabolites or proteins) to manipulate host defensive responses and continue to successfully colonize host tissues. The first extracellular effectors of *M. fijiensis*, MfAvr4 and MfEcp2, were recently identified (Stergiopoulos et al., 2010). MfAvr4 binds fungal chitin and protects the fungal cell wall against plant chitinases. The function of MfEcp2 is not entirely clear, but it probably functions as a necrotrophic factor (Stergiopoulos et al., 2010). These preliminary findings only highlight how very little is known about the apoplastic scenario of the *M. fijiensis-Musa* spp. Interaction. Better understanding, this scenario is vital since it is a potential area for isolation of proteins essential to pathogen establishment, and identification of the plant proteins involved in fungus rejection.

An initial attempt to analyze intercellular proteins from healthy and *M. fijiensis*-infected leaves of *Musa acuminata* ‘Grande Naine’ (AAA) produced only scarce and denatured proteins, undermining any claim of their biological importance (Mendoza-Rodríguez et al., 2006). The present study objective was to recover and analyze native proteins from the apoplast and symplast of healthy, asymptomatic (but infection-positive) and symptomatic diseased *M. acuminata* ‘Grande Naine’ leaves. Electrophoresis analyses were done of enzymatic activities (that is, β-1,3-glucanases, chitinases and proteases) associated with plant defense in this system.

MATERIALS AND METHODS

Plant material

M. acuminata ‘Grande Naine’ plants were grown in nurseries. At pre-fructification stages (approximately 8 months of age), visual criteria (Foure, 1987) were used to identify them as BS-negative (healthy) or BS-positive. *M. fijiensis* infection was confirmed using polymerase chain reaction (PCR) by amplification of the β-tubulin gene of *M. fijiensis.* With these criteria, plants were classified as healthy (absence of *M. fijiensis*; that is, neither visible symptoms of *M. fijiensis* induced lesions, nor PCR amplification of the *M. fijiensis* β-tubulin gene); BS-asymptomatic (early stage with no visible BS symptoms, but positive for the *M. fijiensis* β-tubulin gene by PCR); and BS-diseased plants (visible lesions from BS disease, including stages 1, 2, 3 and 4, and positive for the *M. fijiensis* β-tubulin gene

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Abbreviations: BS, Black Sigatoka; **PAGE,** polyacrylamide gel electrophoresis; **Ri,** retention index; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **PCR,** polymerase chain reaction; **CTAB,** cetyltrimethyl ammonium bromide; **PVPP,** polyvinyl polypyrrolidone; **G6PD,** glucose 6-phosphate dehydrogenase.

by PCR) (Foure, 1987). The selected leaves (sixth or seventh) from healthy or BS-diseased plants were individually sanitized *in planta*, using the method described for recovering of apoplast and symplast, immediately excised from plants, and then processed for each one of the specific analysis.

DNA extraction

Extraction of genomic DNA (gDNA) from healthy, BS-asymptomatic and BS-diseased banana leaves was carried out according to Weising et al. (1991), using the cetyltrimethyl ammonium bromide (CTAB) method. Briefly, pellets were suspended in 50 µL sterile distilled water, the gDNA (1 µg) treated with 50 ng/µL RNAse A, and the quality and quantity ratio of DNA determined by electrophoresis on 1% agarose gels and at 260/280 nm absorbance using a spectrophotometer (Genesys 10 UV).

Amplification of the *M. fijiensis* β-tubulin gene

The PCR reactions were run using gDNA (25 ng) from the banana leaves as template. Each reaction tube contained 3 µL 5x PCR buffer; 0.6 µL 50 mM $MgCl_2$; 0.12 µL Taq Tango; 0.3 µL 10 mM dNTPs; 0.5 µL 10 µM primer β-tubMfF 5'-cgacacagcaagagcagcttc-3'; 0.5 µL 10 µM primer β-tubMfR 5'-ttcgaaagccttggcacttcaa-3'; and sterile distilled water. Total reaction volume was 25 µL. Conditions for PCR were 94°C for 5 min and 35 cycles x [94°C for 40 s, 60°C for 45 s, 72°C for 40 s]; and 72°C for 8 min. The resulting PCR products were separated by electrophoresis on 1% agarose-ethidium bromide gel and viewed on a Gel Doc documentation system (Bio Rad) under UV light.

Apoplast and symplast recovery

The sixth or seventh leaf of selected plants (five leaves per condition) was individually sanitized using a piece of cotton previously soaked with 70% ethanol, and then rinsed with distilled water. Sanitized leaves were excised from the plants and immediately transported to the laboratory. Central veins were removed with a scalpel and leaves cut into homogenous sections. Each condition group (healthy, BS-asymptomatic and BS-diseased) was individually vacuum infiltrated for 1 h under negative pressure at 25 mm Hg using apoplast buffer (AB; 100 mM Tris-HCl [pH 8.0] with added 10 mM ascorbic acid, 5 mM dithiotreitol, 5 mM phenylmethylsulfonyl fluoride, and 3% polyvinyl polypyrrolidone [PVPP]). Apoplastic proteins were recovered by centrifuging at 10,000 x *g* and 4°C; the eluate was called "apoplast". The apoplast-free tissues were then extracted by maceration in a pre-chilled mortar and pestle in the same buffer. The resulting homogenates were centrifuged at 16,000 x g for 10 min; the supernatants were called "symplast". Symplast samples were stored at -20°C until use.

Protein precipitation

For protein enrichment, the apoplasts were independently precipitated with acetone at a final concentration of 8%, incubated at -20°C for 2 h and then centrifuged at 16000 x g for 10 min at 25°C. The supernatants were discarded and the pellets dried at 25°C for 20 min. Pellets from ten tubes were suspended in 10 µL AB buffer supplemented with 1% glycerol and stored at -20°C until use.

Protein quantification

Protein concentration was determined according to Peterson (1977) at 750 nm absorbance, using bovine serum albumin and fraction V as a protein standard.

Protein electrophoresis

Protein preparations (20 µg each) were separated on 12% denaturing gels (SDS-PAGE) according to Laemmli (1970), and on 12% native gels (PAGE). Polypeptides were viewed by staining the gels for total protein using silver or for enzymatic activity (described below).

Zymography of β-1,3-glucanases, chitinases and proteases

For zymographic analyses, native 12% polyacrylamide gels were loaded with samples recovered from apoplastic fluids of healthy (1), BS-asymptomatic (2), and BS-diseased (3) leaves or with symplastic fluids from healthy (5), BS-asymptomatic (6), and BS-diseased (7) leaves. Gels were electrophoresed for 3 h at 100 V, and 25°C. The β-1,3-glucanase activity was assayed according to Pan et al. (1991). Briefly, the gels were individually incubated in sodium acetate (50 mM; pH 5.0) for 5 min immediately after the PAGE, and the solution discarded. The gels were then incubated at 40°C for 60 min in 25 mL 50 mM sodium acetate (pH 5.0) with addition of 0.15 g laminarin previously dissolved in 25 mL H_2O. Finally, they were submerged in methanol:water:acetic acid (5:5:2; V/V/V) for 5 min and washed in deionized water for 30 s. The activity of β-1,3-glucanase was viewed by incubating the gels in 30 mL triphenyl tetrazolium chloride (0.15 g/50 mL 1.0 M NaOH) and warming in a microwave oven five times, 10 s each time. Activity bands appeared as purple bands on a pink background. Beta-1,3-glucanase from *Helyx pomatia* (Sigma) was included in the gel as a positive control.

Chitinase was identified by co-polymerizing 12% native gels in the presence of 0.01% glycol-chitin (Sigma). Gels were loaded with protein from each sample, and after electrophoresis were washed for 30 min in 100 mM sodium acetate (pH 5.0). The washing buffer was exchanged with 50 mL sodium acetate (100 mM [pH 5.0], with added 1% Triton X-100) and incubated for 22 h at 37°C. The gels were then transferred to 50 mL 500 mM Tris-HCl (pH 8.9) with added 0.05% Brightener 28 (5 min), and washed 5 times in distilled water. Chitinase activity was viewed as dark bands on a white background by using UV light in a Gel Doc device. Commercial chitinase from *Streptomyces griseus* (Sigma) was used as a positive control.

Protease identification was done according to Distefano et al. (1997). Briefly, native gels were co-polymerized in the presence of 2% gelatin, and incubated for 60 min at 37°C in 1% Triton X-100. This solution was exchanged with 2.5% Triton X-100 solution and incubated at 37°C for 60 min, followed by an overnight incubation at 37°C in 50 mL 250 mM Tris-HCl (pH 7.5). Enzymatic activity was viewed by staining the gels with Coomassie blue to produce clear bands on a blue background. Trypsin II-6 (10 µg) from porcine pancreas (Sigma) was used as a positive control. Retention index (Ri) values for all enzyme migrations were calculated using the ratio: Ri = distance (mm) migrated by enzyme in gel / total distance (mm) migrated in gel by front of samples.

Determination of enzymatic activity of glucose 6-phosphate dehydrogenase

Glucose 6-phosphate dehydrogenase (G6PD) activity was assayed in 750 µL of buffer containing 100 mM Tris-HCl (pH 8.0), with added 10 mM $MgCl_2$, 6 mM glucose 6-phosphate and 4 mM NADP (200 µL). The mixture was incubated at 25°C for 5 min and the reaction initiated by adding 10 µL protein sample (3 µg protein) in 950 µL

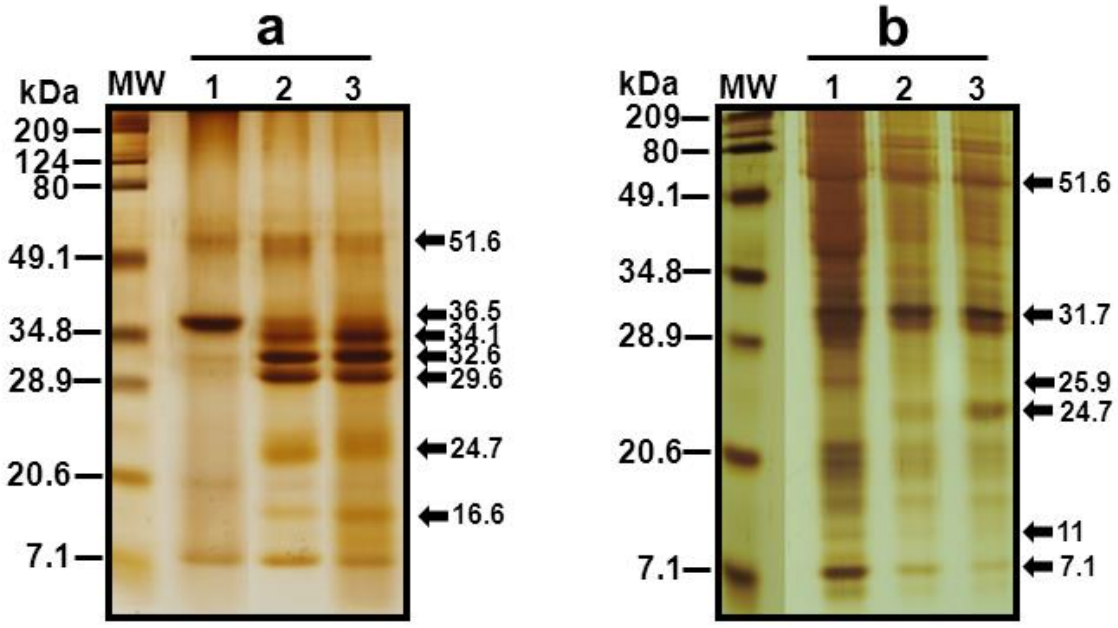

Figure 1. Apoplastic and symplastic polypeptides from *Musa acuminata* and the *Musa-acuminata-Mycosphaerella fijiensis* interaction. Protein samples were resolved on 12% SDS-PAGE gels. Fluids were from healthy (a1), BS-asymptomatic (a2), and BS-diseased (a3) apoplast, or from healthy (b1), BS-asymptomatic (b2), and BS-diseased (b3) symplast. Arrows to the right indicate polypeptides that remained unchanged, appear, or disappear as disease symptoms progressed. MW corresponds to sizes of protein molecular weight standards.

total volume. Enzyme activity was monitored at 25°C by following increases in absorbance at 340 nm for 15 min. Specific activity was calculated using the extinction coefficient of NADP (6.18 mM cm^{-1}), and expressed as µmol substrate reduced per min^{-1} g of $tissue^{-1}$.

Evaluation of banana tissue integrity

Leaf disks from healthy, BS-asymptomatic and BS-diseased leaves (three replicates each), were infiltrated with extraction buffer and then bleached at 8°C in ethanol-chloroform (4V/1V) for 18 days. Bleached samples were transferred to 70% ethanol and incubated overnight at 4°C. Samples were then transferred to 50% glycerol-ethanol and incubated for an additional two days at 4°C. After incubation, the samples were covered with a 0.01% trypan blue solution for 20 min and observed with a normal transmitted light microscope (Carl Zeiss). Images were taken using a digital camera (Kodak).

RESULTS

Apoplast and symplast analyses

SDS-PAGE analysis identified few polypeptides in the apoplastic fluid from healthy banana plants. The most abundant had a molecular weight of 36.5 kDa (Figure 1a, lane 1), and was notably less abundant in apoplastic fluids from BS-asymptomatic and BS-diseased plants (Figure 1a, lanes 2 and 3, respectively). Three polypeptides (molecular weights = 34.1, 24.7 and 16.6 kDa) were present in apoplastic fluid from BS-asymptomatic and BS-diseased plants, but absent in fluid from healthy plants (Figure 1a, lanes 2 and 3). A third group of polypeptides (MW = 32.6 and 29.6 kDa) was barely present in the healthy tissue, but quite notable in the BS-asymptomatic and BS-diseased tissues (Figure 1a, lanes 1, 2 and 3). In the symplastic fluid, a complex pattern of polypeptides ranging from 2.1 to 209 kDa was observed in healthy plants (Figure 1b, lane 1). Levels of these polypeptides were generally lower in BS-asymptomatic and BS-diseased plants. The 32.6, 25.9, 20.6, 11 and 7.1 kDa polypeptides decreased in abundance in comparison with levels in the healthy symplast (Figure 1b, lanes 2 and 3). In contrast, a small number of polypeptides (24.7 kDa) increased in the BS-diseased symplastic fluid (Figure 1b, lanes 2 and 3), while some polypeptides (51.6 kDa) remained relatively unchanged in all conditions (Figure 1b). Protein profiles dynamics are summarized in Figure 6.

Zymography of β-1,3-glucanase, chitinase and protease

Plants use β-1,3-glucanase and chitinase enzymes to efficiently control fungal pathogens (Floerl et al., 2008; Shetty et al., 2009; Pan et al., 1991). The possible presence of enzymatic defensive weapons in *M.*

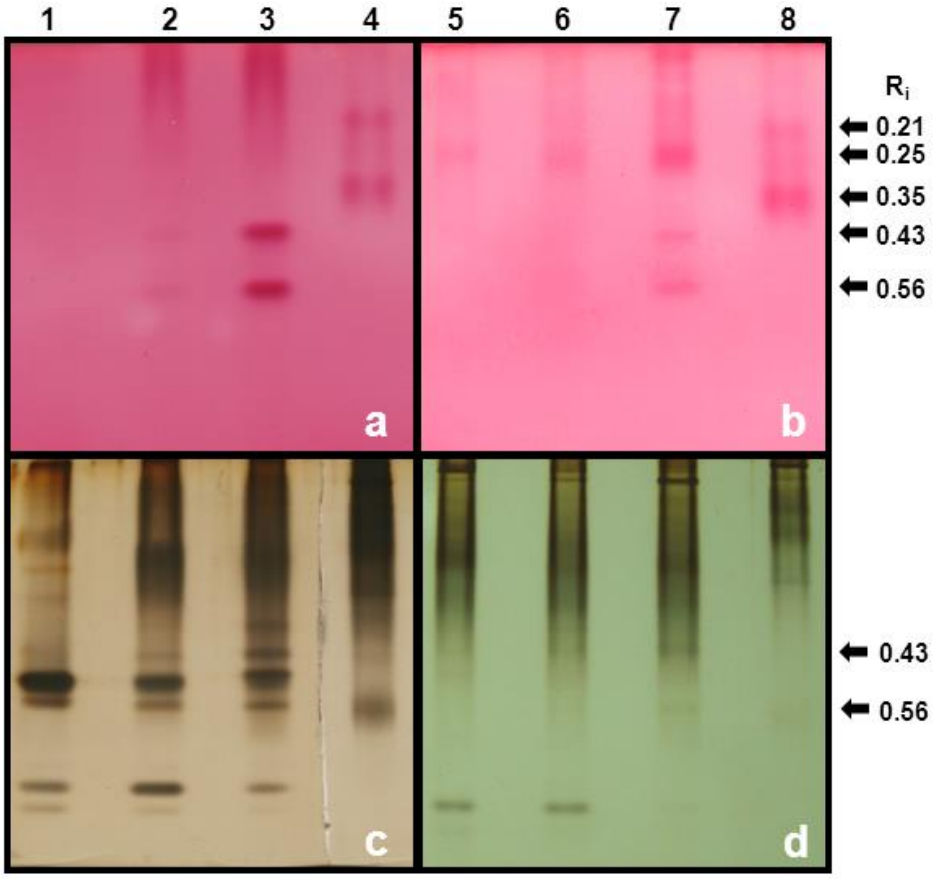

Figure 2. Zymography in non-reducing 12% polyacrylamide gels of β-1,3-glucanase from apoplastic and symplastic fluids of *Musa acuminata* and the *M. acuminata-M. fijiensis* interaction. Beta-1,3-glucanase was analyzed in apoplastic fluids recovered from healthy (a1), BS-asymptomatic (a2), and BS-diseased (a3) leaves, or in symplastic fluids from healthy (b5), BS-asymptomatic (b6), and BS-diseased (b7) leaves. Commercial (Sigma) β-1,3-glucanase from *Helix pomatia* (lanes a4 and b8) was used as a positive control. Arrows to the right indicate R_i of bands with enzymatic activity (a and b), and silver-stained polypeptides (c and d) that co-migrated at the same position where enzymatic activity was detected.

acuminata versus *M. fijiensis* attack was tested by analyzing β-1,3-glucanase enzymatic activity in apoplastic and symplastic fluids, with and without fungal challenge. Enzymatic activity was scarce in apoplastic fluid from healthy plants but more detectable as bands of variable intensity with Ri = 0.43 and 0.56, in apoplastic fluids from BS-asymptomatic plants (Figure 2a, lane 2) and BS-diseased plants (Figure 2a, lane 3). In general, symplastic fluids exhibited similar results with strong β-1,3-glucanase activity detected in BS-diseased tissues (Figure 2b, lanes 3 and 7, respectively). A band with a Ri = 0.25 was observed in the symplast of healthy and BS-diseased plants but with the strong signal in the last (Figure 2b, lanes 5, 6 and 7). The positive control, a commercial β-1,3-glucanase from *H. pomatia* (Sigma), exhibited two polypeptides (R_i = 0.21 and 0.35) that produced clear activity signals (Figure 2a and b, lanes 4 and 8, respectively). In all samples, β-1,3-glucanase enzymatic activity bands correlated with protein bands in the PAGE gels (Figure 2c and 2d).

Chitinases in apoplastic fluids (Figure 3a, lanes 1 to 3), exhibited activity associated with two bands of R_i = 0.63 and 0.89. The 0.63 band decreased in activity in fluids from BS-asymptomatic and BS-diseased plants, while the 0.89 band increased in intensity in BS-asymptomatic and BS-diseased plants (Figure 3a, lanes 1, 2, 3). Chitinase activity in symplastic fluids was associated with three bands showing R_i = 0.58, 0.63 and 0.89. Unlike in apoplastic fluids, the 0.63 band remained constant in healthy and BS-asymptomatic symplastic fluids, and was negligible in BS-diseased fluids (Figure 3b, lanes 5, 6, 7). The 0.89 band progressively increased intensity from healthy to BS-diseased samples (Figure 3b, lanes 5, 6, 7). The 0.58 band was visible only in symplast from BS-

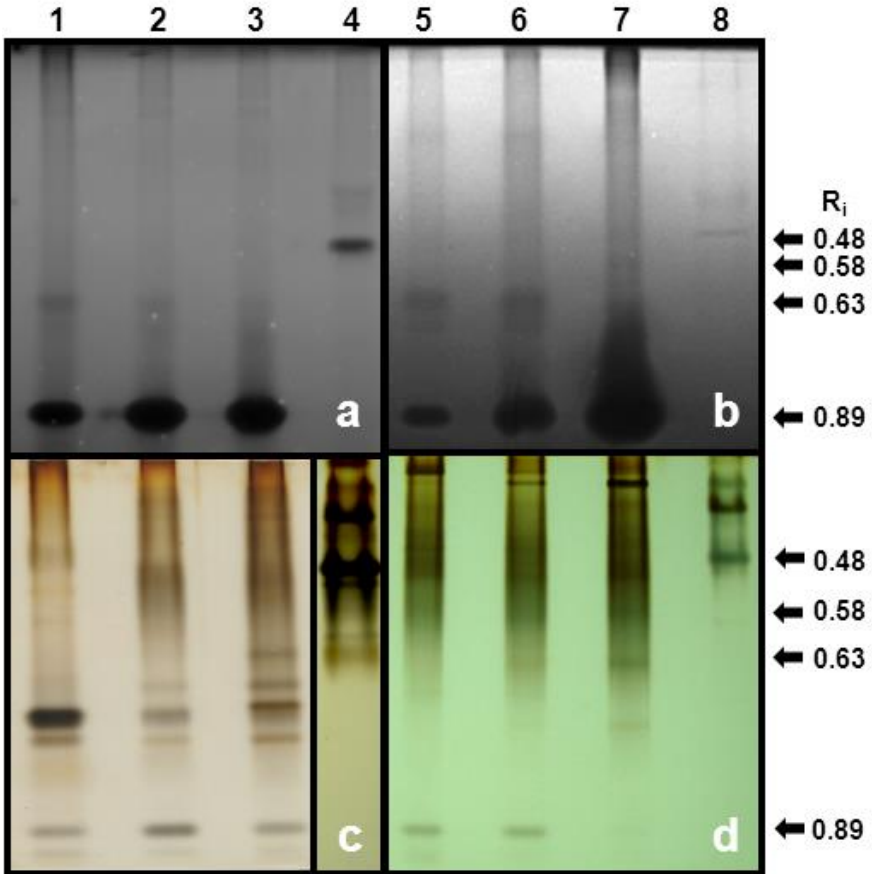

Figure 3. Zymography in non-reducing 12% polyacrylamide gels of chitinase enzymatic activity in apoplastic and symplastic fluids of *Musa acuminata* and the *M. acuminata-M. fijiensis* interaction. Lane order is the same as in Figure 2. Commercial (Sigma) chitinase from *Streptomyces griseus* (a4 and b8) was used as a positive control.

diseased samples (Figure 3b, lane 7). The chitinase activity bands of the apoplastic or symplastic fluids coincided with polypeptides in the equivalent silver-stained gels (Figure 3c and d). The 0.63 polypeptide was most abundant in healthy apoplast, decreased in asymptomatic and diseased apoplast (Figure 3c, lanes 1, 2, 3), and was faint in the symplast samples (Figure 3d, lanes 5, 6 and 7). The 0.89 polypeptide remained constant in all three apoplast conditions (healthy, BS-asymptomatic and BS-diseased) (Figure 3c, lanes 1, 2 and 3), as well as in the healthy and BS-asymptomatic symplast (Figure 3d, lanes 5, 6); it was barely detectable in the BS-diseased symplast (Figure 3d, lane 7). One band with R_i = 0.48 representing chitinase activity was visible in both the positive control (commercial chitinase from *S. griseus*, Sigma) (Figure 3a and b, lanes 4 and 8), and the equivalent silver-stained gel (Figure 3c and d, lanes 4 and 8).

Proteolytic activity was evaluated because it is prominent in the interaction between plants and pathogens (Shabab et al., 2008; Song et al., 2009; Bozkurt et al., 2011; Dixelius, 1994). In the apoplast samples, protease activity was slight and mainly associated with two polypeptides showing R_i = 0.04 and 0.14 (Figure 4a). The 0.14 band was more intense in healthy plant apoplast (Figure 4a, lane 1) than in the BS-asymptomatic and BS-diseased tissues (Figure 4a, lanes 2 and 3, respectively). The contrary occurred with the 0.04 band, which was faint in healthy plant apoplast (Figure 4a, lane 1), but more intense in the BS-asymptomatic and BS-diseased apoplast (Figure 4a, lanes 2 and 3). In the symplastic fluids, proteolytic activity was stronger than in the apoplastic fluids, and was again associated with two bands (R_i = 0.04 and 0.14).

In a pattern similar to that observed in the apoplastic fluids, the 0.14 band progressively decreased in intensity from the healthy tissues to the BS-asymptomatic (Figure 4b, lane 6) and BS-diseased fluids (Figure 4b, lane 7). In contrast, the 0.04 band increased in intensity from the healthy to the BS-asymptomatic and BS-diseased tissues (Figure 4b, lanes 6 and 7, respectively). These data suggest that proteolysis behaves differentially and

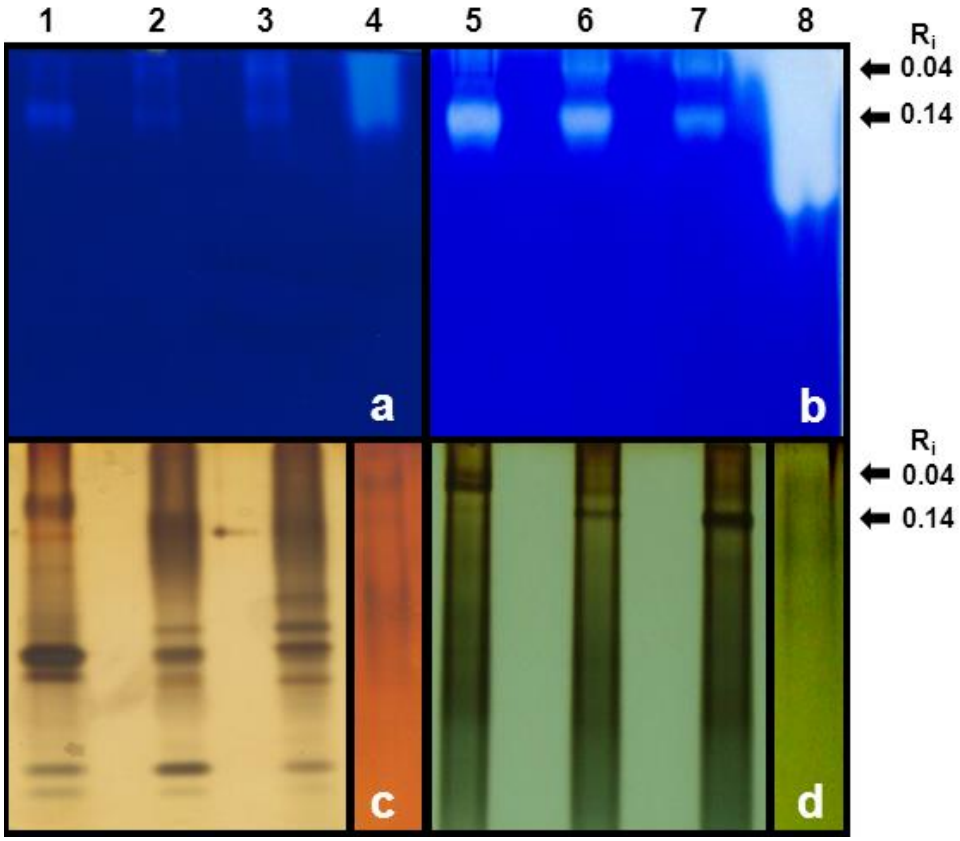

Figure 4. Zymography in non-reducing 12% polyacrylamide gels of protease activity in apoplastic and symplastic fluids from *Musa acuminata* and the *M. fijiensis-Musa acuminata* interaction. Lane order is the same as in Figures 2 and 3. Commercial bovine trypsin II-6 (lanes a4 and b8) was used as a positive control.

dynamically in apoplastic and symplastic fluids during BS disease progression. Commercial Trypsin II-6 from porcine pancreas (Sigma) was used as a positive control for protease activity, and exhibited high proteolytic activity in the upper portion of the lane (Figure 4a and 4b, lane 4 and 8, respectively).

The silver-stained gels exhibited a complex pattern of polypeptides in the apoplastic fluids, with some bands having the same R_i as the putative proteases (Figure 4c, lanes, 1, 2, 3). Two polypeptides co-migrated to the same positions where protease activity bands were observed (Figure 4d, lanes 5, 6, 7).

Glucose 6-phosphate dehydrogenase (G6PD) activity

Since β-1,3-glucanase, chitinase and protease activities were present in both apoplastic and symplastic fluids from all the samples, the specific cytosolic marker, glucose 6-phoshate dehydrogenase (G6PD), was measured to test if apoplast enzymatic activities were caused by loss of cell integrity and/or contamination with cytosolic content. Comparison of specific G6PD activity (µkat gPF^{-1}) in the symplastic and apoplastic fluids identified minimal contamination of apoplast samples with symplast content (Table 1). Therefore, the enzymatic activities detected in the apoplast, especially of β-1,3-glucanase and chitinase, cannot be explained by contamination with symplastic fluid.

Banana leaf tissue integrity

Macro-histology of the banana tissues showed color differences after buffer infiltration and bleaching and trypan blue staining. Healthy tissue remained slightly green (Figure 5a, lane 1), while BS-asymptomatic tissues were slightly brown and BS-diseased tissues were clearly brown (Figure 5a, lanes 2 and 3), suggesting tissue phenolyzation. Micro-histology of the healthy tissue confirmed tissue integrity and the absence of fungal mycelium (Figure 5b, lane 1), while the BS-asymptomatic and BS-diseased tissues clearly exhibited affectation. In addition, mycelium was observed in the host extracellular space in the BS-diseased tissue (Figure 5b, lane 3). Tissue directly stained with trypan blue for 20 min

Table 1. Comparison of glucose 6-phosphate dehydrogenase activity in apoplastic and symplastic fluids of *Musa acuminata* and the *Musa acuminata-M. fijiensis* interaction.

Sample	Enzymatic activity (µkat/g fresh weight)	Times of enzymatic activity symplast/apoplast
Healthy symplast	2.5056	57.75
Healthy apoplast	0.04338	
Asymptomatic symplast	0.3791	51.6
Asymptomatic apoplast	0.00734	
Diseased symplast	0.2562	34.9
Diseased apoplast	0.00734	

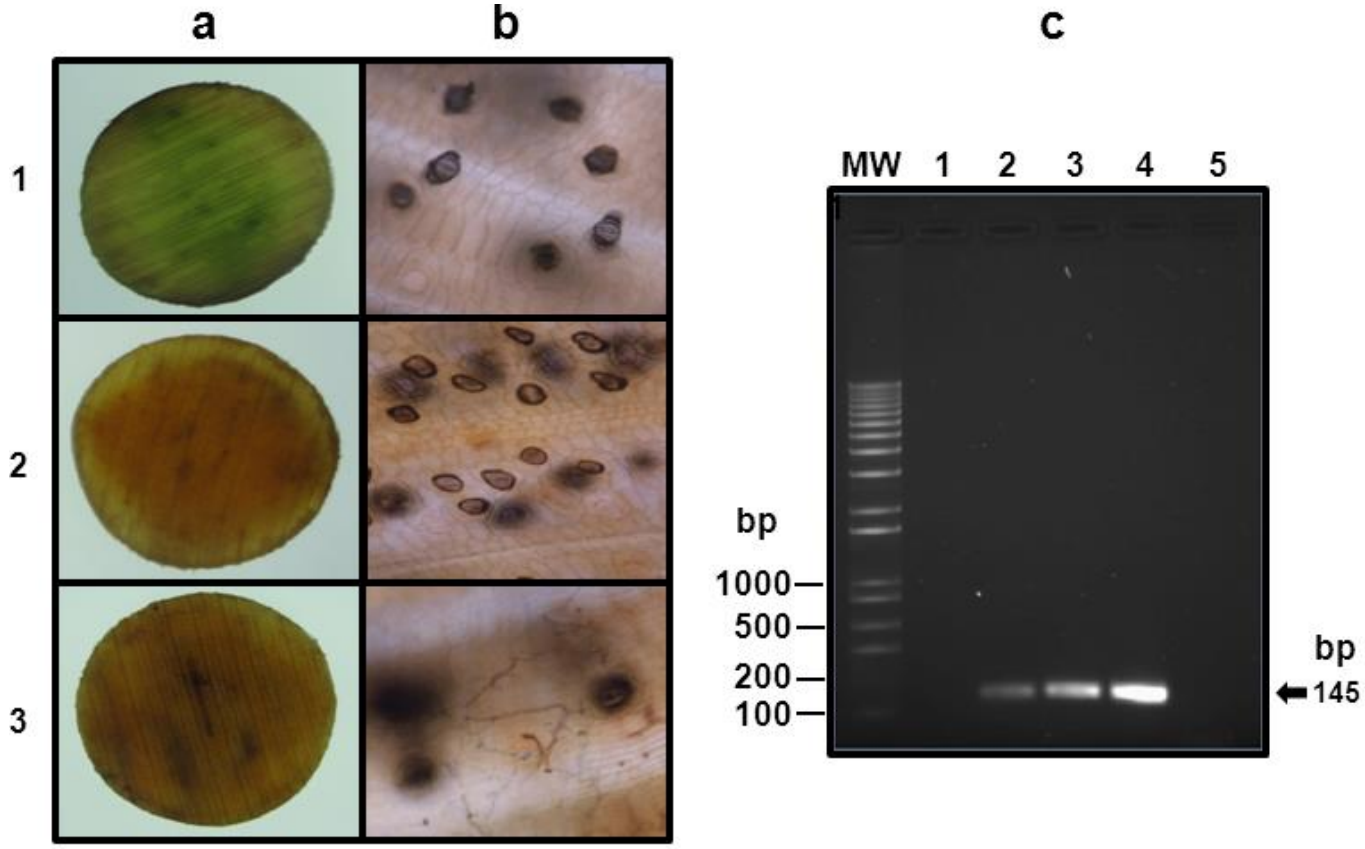

Figure 5. Macro (panel a) and micro-histological (panel b) observations of *Musa acuminata* 'Grande Naine' leaf tissues and analysis on 1% agarose gels of the PCR amplified β-tubulin gene from *M. fijiensis* (c). Leaf disks were from infiltrated healthy (a1), BS-asymptomatic (a2), or BS-diseased (a3) leaf tissues. Microhistology images are of infiltrated healthy (b1), BS-asymptomatic (b2), or BS-diseased (b3) leaf tissues. The specific PCR and separation of its products on a 1% agarose gel show results for DNA samples from healthy (c1), BS-asymptomatic (c2), and BS-diseased (c3) leaves. Genomic DNA (gDNA) of *M. fijiensis* was used as a positive control (c4), and water as a negative control (c5). MW = 1 kb DNA ladder.

showed that all samples (that is, healthy, BS-asymptomatic and BS-diseased leaves) excluded the dye. This indicates that at the time the cells still had sufficient integrity to prevent trypan blue uptake, suggesting the possibility of cell controlled protein uploading into the apoplastic space.

PCR amplification of the *M. fijiensis* β-tubulin gene

A PCR diagnosis was run to support visual classification of the banana leaf tissue samples into healthy, BS-asymptomatic and BS-diseased conditions. Lack of amplification of the 145 bp-fragment of the *M. fijiensis* β-

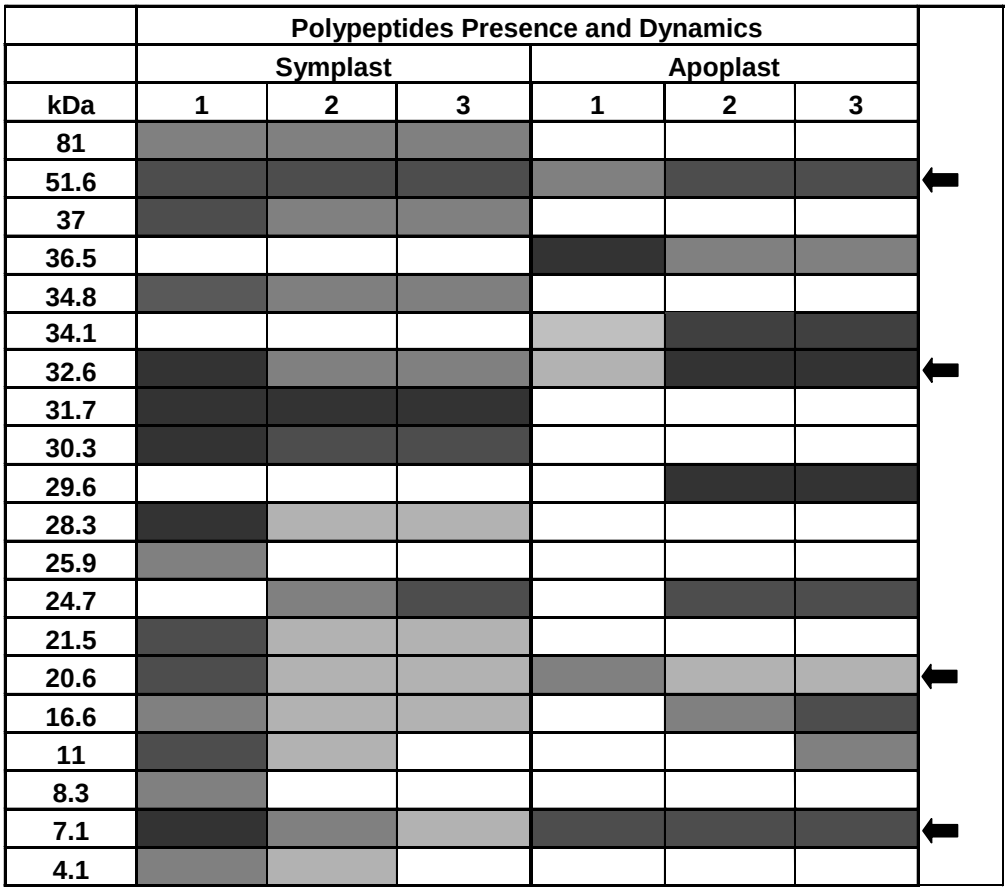

Figure 6. Schematic representation of polypeptides from the apoplastic and symplastic fluids of *Musa acuminata* 'Grande Naine' and the *M. acuminata-M. fijiensis* interaction. Polypeptides were obtained from apoplastic or symplastic fluids collected from healthy (1), BS-asymptomatic (2), and BS-diseased (3) banana leaves. Tone intensity represents relative polypeptide abundance: black = high abundance; dark gray = moderate abundance; light gray = low abundance; white = absent. Arrows indicate polypeptide bands present in all samples.

tubulin gene confirmed absence of the pathogen in the healthy sample (Figure 5c, lane 1). This product was readily amplified in the BS-asymptomatic and BS-diseased tissues (Figure 5c, lanes 2 and 3, respectively). The analysis included a *M. fijiensis* gDNA template as positive control (Figure 5c, lane 4), and no template as a negative control (Figure 5c, lane 5).

DISCUSSION

Protein patterns in apoplast and symplast fluids

Plant apoplast is a dynamic compartment inside the organism with a broad range of physiological functions; for example, nutrition, growth, signaling, transport, plant defense, and plant-pathogen interaction, among others (Alves et al., 2006). The present study follows up on pilot research by Mendoza-Rodríguez et al. (2006). They began focusing on isolation of apoplastic proteins from the *M. fijiensis-M. acuminata* interaction, but obtained very low amounts of denatured proteins. The present results represent the first time proteins have been recovered from apoplastic compartment in an active form. Proteins in the apoplastic fluid were suitable for zymography of β-1,3-glucanases, chitinases and proteases, demonstrating that they retained their functional structure. This clearly illustrates this method's effectiveness in recovering native proteins from the *M. acuminata-M. fijiensis* interaction. Protein analysis by SDS-PAGE and PAGE identified clear differences in polypeptide profiles and zymographies between the apoplastic and symplastic preparations. The SDS-PAGE results showed accumulation of specific polypeptides (that is, 34.1, 24.7 and 16.6 kDa) in the BS-infected apoplast (Figures 1 and 6), strongly suggesting that these proteins originated in the host. These proteins were probably the result of *de novo* expression triggered by the

plant's pathogenic response since they were absent in healthy tissues. Under microscopic examination, the BS-asymptomatic samples contained no observable fungal tissue, indicating that fungal material was scarce (Figure 5, panel 2b).

Zymography of β-1,3-glucanase, chitinase and protease activity

Observation of putative pathogen-inducible β-1,3-glucanases with Ri = 0.43 and 0.56 in the apoplastic fluids of BS-infected tissues supports a putative role of these enzymes in the response of *M. acuminata* to *M. fijiensis*. Dixelius (1994) described a β-1,3-glucanase in *Brassica napus* which was uploaded in the apoplastic space of leaves after infection with *L. maculans*. He demonstrated that β-1,3-glucanase in the apoplast was not associated with cellular damage during fungal growth or with contamination of apoplastic fluid. Also, in *Brassica rapa*, β-1,3-glucanase gene expression was rapidly triggered after inoculation with an avirulent strain of *Xanthomonas campestris*, with maximum production at 24 h after inoculation (Newman et al., 1994). Apoplastic localization of β-1,3-glucanase has also been reported in other plant-fungal interactions (*C. fulvum*-tomato (Joosten and De Wit, 1989); *Venturia inaequalis*-apple 'Remo' (Gau et al., 2004). The present study addresses presence of β-1,3-glucanase in healthy, BS-asymptomatic (biotrophic stage), and BS-symptomatic (necrotrophic stage) leaf tissue in a compatible interaction of *M. acuminata* with *M. fijiensis*. Study results are consistent with similar reports in other pathosystems. Beta-1,3-glucanase accumulated in the necrotrophic phase since no enzymatic activity was observed in healthy tissues and was represented by only faint bands in the BS-asymptomatic tissues. This suggests that the compatible interaction plant response in the form of β-1,3-glucanase expression, is insufficient and thus unable to contend with the fungal infection. Torres et al. (2012) recently reported induction of different pathogenesis-related proteins in banana in response to *M. fijiensis* infection. They identified two peaks in β-1,3-glucanase activity in both the compatible interaction with *M. acuminata* 'Williams', and the incompatible interaction with *M. acuminata* 'Calcutta 4'. However, the time required to trigger the response was shorter in the 'Calcutta 4' (first peak at 6 h post-infection) than in the 'Williams' (18 h post-infection). Maximum β-1,3-glucanase activity was similar in both cultivars, but appeared earlier in 'Calcutta 4'. In both cultivars maximum activity peaks were triggered quickly (that is, <72 h post infection). Despite these similarities, the 'Williams' displayed symptoms nine days after infection, while the 'Calcutta 4' exhibited only small necrotic lesions after 72 h, and successfully arrested the pathogen. Given these results, it can be expected that differences in the responses of susceptible and resistant hosts could be both temporal and spatial. However, these authors assayed β-1,3-glucanase activity in total leaf homogenates, making it impossible to associate the activity with symplastic or apoplastic compartments, or with local leaf zones. Further research assessing these differences in β-1,3-glucanase expression will help to build a more complete picture of β-1,3-glucanase's role in the banana-*M. fijiensis* interaction.

Chitinase is found frequently in xylem sap and apoplastic fluids during plant-pathogen interactions. Apparently, it acts either independently against fungal attack, or in a concerted way with β-1,3-glucanases, by weakening the pathogen cell wall. Chitinases have been described in the interactions of *Verticillum longisporum-Arabidopsis thaliana* (Johansson et al., 2006); *V. longisporum-B. napus* (Floerl et al., 2008); *S. tritici-Triticum aestivum* L. (Shetty et al., 2009; Segarra et al., 2003); and *C. fulvum-Lycopersicon esculentum* (Joosten and De Wit, 1989), among others. In the present results, a band with R_i = 0.89, associated with chitinase activity was more intense (that is, active) in apoplastic and symplastic fluids from BS-diseased *M. acuminata* leaves and fainter (weaker) in fluids from healthy leaf tissue.

Sánchez-García et al. (2012) recently reported the presence of chitinase activity from leaf homogenates of infected banana 'Grande Naine' and 'Calcutta 4'. Response was faster and stronger in 'Calcutta 4' than in 'Grande Naine'. They evaluated chitinase activity only during the first 6 days post-infection, when leaves in the compatible interaction are still asymptomatic. In contrast, Torres et al. (2012) measured chitinases throughout the entire interaction (in the compatible interaction in 'Williams') in leaf homogenates. Their results were similar to those of Sánchez-García et al. (2012) in that strong chitinase activity peaks were apparent at an early interaction stage. At a later stage, a second large, broad peak in activity was observed; in this case, chitinase activity was higher during the asymptomatic stage than in the necrotrophic stage. The present results also support a slow or delayed response of the susceptible 'Grande Naine' host but, unlike in Torres et al. (2012), chitinase activity was highest during necrotrophy. This discrepancy may be due to use of a different susceptible host, or that the kinetics of BS disease progress is not identical in all cases. All the above results suggest that response timing is key to determining host capability to cope with or limit pathogen attack (Mendoza-Rodríguez et al., 2006).

No previous reports exist of proteolytic activity during the banana-*M. fijiensis* interaction. Our group has previously described detection of strong protease activity in the *in vitro* secretome of *M. fijiensis* (Chuc-Uc et al., 2011). In addition, proteolysis and proteases are reported to have important roles in other pathosystems (*S. tritici-Triticum aestivum* (Shetty et al., 2009); *Phytophthora infestans-Carica papaya* (Odani et al., 1996); *Phytophthora infestans-Lycopersicon esculentum*

(Valueva et al., 1998); and *Phytophthora infestans*-*Nicotiana benthamiana* (Bozkurt et al., 2011), particularly by regulating host serine protease activity (Segarra et al., 2003). In the present study, a dual behavior was observed for polypeptides displaying protease activity. On the one hand, the R_i = 0.04 polypeptide increased its activity in the apoplast and symplast of BS-infected samples. Our group has reported that proteases may participate in development of foliar lesions (Chuc-Uc et al., 2011), and therefore this polypeptide may be involved in the progress of BS disease symptoms. On the other hand, the R_i = 0.14 polypeptide displayed a high level of activity in apoplastic and symplastic fluids from healthy plants, with declining activity in infected tissues with disease progression. This suggests negative regulation which may or may not be due to presence of *M. fijiensis*. Inhibition of host proteases has been described as a pathogen strategy to inactivate host defenses. For example, in the host *Solanum lycopersicum*, *C. fulvum* inhibits a defense-related apoplastic Cys protease (Shabab et al., 2008), as well as the Rcr3 protease (Song et al., 2009). *Phytophthora infestans* also blocks a host protease to infect *Nicotiana benthamiana*, and in papaya it inhibits apoplast secretion of a papain-like cysteine protease C14, causing its peripheral accumulation inside the symplast (Song et al., 2009). In the present case, therefore, *M. acuminata* proteases may also be targeted by *M. fijiensis* during infection.

Glucose 6-phosphate dehydrogenase (G6PD) enzymatic activity

Apoplastic preparation quality is estimated by enzymatic measurement of specific proteins such as malate dehydrogenase (Stergiopoulos et al., 2010; Tasgin et al., 2006), or glucose-6-phosphate dehydrogenase (Tasgin et al., 2006). In the present study, glucose-6-phosphate dehydrogenase was used as a marker for apoplastic purity because it is generally accepted that this enzyme has an exclusively cytosolic localization. Its detection in apoplastic fluid is therefore indicative of apoplastic contamination with cell cytoplasmic contents. Based on this criterion, the apoplastic fluid preparations had only a 2 to 3% symplastic fluid contamination level (Table 1), meaning the majority of recovered fluid was apoplastic. Contamination with symplastic fluid occurs frequently due to damage caused during tissue sampling; this is supported by detection of trypan blue-stained cells surrounding the sampled area at tissue sampling sites (data not shown). Considering the high purity of the analyzed apoplastic fluid, it can be confidently stated that β-1,3-glucanase, chitinase, and protease activities identified here occurred in the extracellular space of the sampled banana leaves. In other words, apoplastic proteins were successfully recovered in their native state. This procedure opens the door for new and interesting research possibilities in this pathosystem. The present results on black Sigatoka disease in banana will prove valuable in developing control strategies against *M. fijiensis* in addition to development of resistant plants. Proteins occurring in the apoplastic space during the plant-pathogen interaction dynamic play important roles and are specifically secreted by the host for plant defense, or by the pathogen to elude plant defenses. For instance, in *Malus domestica* 'Remo', pathogen resistance is associated with occurrence of a number of pathogenesis-related proteins in the apoplast (Gau et al., 2004). Knowledge of these protease activities suggests development of a black Sigatoka disease control strategy in *M. acuminata* 'Grande Naine' in which plants could be stimulated to constantly produce PR proteins, resulting in secretion in the apoplastic space at levels similar to those found in resistant varieties. This could reduce dependence on fungicides, with consequent financial advantages for producers.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Carotenoids from *Phaffia rhodozyma*: Antioxidant activity and stability of extracts

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The main goal of this work was to establish the stability and antioxidant activity of the extracts obtained through different techniques for recovering carotenoids from *Phaffia rhodozyma* NRRL-Y 17268. The best conditions for extracting carotenoids through cell rupture with dimethylsulfoxide (DMSO) were found to be a particle size of 0.125 mm submitted to freezing temperature (-18°C) for 48 h (272 µg/g). For DMSO extracts, freezing negatively affected the antioxidant activity by 2,2 '-azinobis (3-ethyl benzothiazoline-6-sulfonic acid)) and DPPH (2,2-diphenyl-1-picrylhydrazyl (DPPH) methods. The carotenogenic extracts obtained by enzymatic disruption proved to be more promising in relation to its antioxidant activity.

Key words: Microbial carotenoids, antioxidant properties, cell wall disruption.

INTRODUCTION

Carotenoids are widespread in nature and found in several plants, animals and microorganisms (Maldonade et al., 2008). According to BCC Research (2008), the global market for carotenoids was estimated at about $1.2 billion in 2010, with the potential to grow to $1.4 billion in 2018. They are mostly produced by chemical synthesis. However, the biotechnological production of these pigments by yeasts has been highlighted for possibly using low cost substrates in cultivation, control of metabolites with biological activity, designation of natural substances, the small space required for production, independence of environmental conditions such as weather, season or soil composition, and control of culture conditions (Zeni et al., 2011).

The yeast *Phaffia rhodozyma*, also known as *Xanthophyllomyces dendrorhous*, stands out as a natural source of carotenoids. It has a pattern of relatively rapid growth and nutritional quality as well as being approved as a Generally Recognized as Safe (GRAS) microorganism by the Department of Health and Human Services of the Food and Drug Administration (FDA, 2000). *P. rhodozyma* produces different carotenoids depending on the growth conditions. This yeast produces astaxanthin in its configuration (3R, 3'R), and so far it is the only known

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natural source of this stereoisomer. It is also capable of producing β-carotene, which is a dicyclic carotene produced from neurosporene through lycopene (An et al., 1999; Rodriguez-Amaya, 2001; Grewe et al., 2007; Schmidt et al., 2010; Chang et al., 2015; Xiao et al., 2015).

Several potential vegetable sources of carotenoids with antioxidant activity have been studied, such as mango wine (Varakumar et al., 2011), tomatoes (Li et al., 2012), carrots, green beans and broccoli (McInerney et al., 2007), as well as the macrofungi *Phenillinus merrilli* (Chang et al., 2007). However, few studies have been conducted on the antioxidant activity of carotenoids obtained from microbial sources.

On the other hand, different cell disruption methods are available for carotenoid recovery from yeast biomass, including chemical, physical and enzymatic techniques (Michelon et al., 2012). Furthermore, some works have demonstrated that biomass freezing can improve extraction yield (Fonseca et al., 2011). However, there is no information regarding the effects of these treatments on the antioxidant activity of carotenogenic extracts.

In this context, the main goal of this study was to establish the antioxidant activity and stability of the carotenogenic extracts from *P. rhodozyma* NRRL-Y 17268. For this, the effect of particle diameter of biomass on carotenoid recovery was evaluated, and its stability during freezing was taken into consideration. Moreover, antioxidant activities of extracts obtained by chemical and enzymatic techniques of cell disruption were evaluated.

MATERIALS AND METHODS

Microorganism, maintenance and reactivation

This study used the yeast, *P. rhodozyma* NRRL-Y 17268 supplied by Northern Regional Research Laboratory (Peoria, USA). Prior to the experiments, the yeast was maintained at 4°C on yeast malt (YM) agar supplemented with 0.2 g/L of KNO_3 with the following composition (g/L): 3 yeast extract; 3 malt extract; 5 peptone; and 10 glucose (Parajó et al., 1998). For reactivation of stock cultures, transfers to tubes containing the same medium were made and the tubes were incubated for 48 h at 25°C. They were scraped with 10 mL of 0.1% (w/v) peptone diluent for each tube and transferred to 500 mL Erlenmeyer flask (Santos et al., 2012) containing YM medium (3 g/L yeast extract; 3 g/L malt extract; 5 g/L peptone; and 10 g/L glucose) and incubated under the same conditions.

Inoculum

The suspension obtained (10 mL) was transferred to a 500 mL Erlenmeyer flask containing 90 mL YM broth supplemented with 0.2 g/L of KNO_3 (Grewe et al., 2007). The suspension was maintained at 25°C in a rotary shaker at 150 rev/min for 48 h or the time required to achieve $1x10^8$ cells/mL by counting in a Neubauer chamber (Zhang et al., 2005).

Shaken flask cultivation

The cultivation medium was inoculated with 10% (v/v) of inoculum (reaching 10^7 cells/mL). The cultivation was carried out in 500 mL Erlenmeyer flasks containing 153 mL YM medium on a rotary shaker (180 rev/min) at 25°C for 168 h (Fonseca et al., 2011). The initial pH was adjusted to 6.0 and the medium was sterilized at 121 °C for 15 min.

At the end of cultivation (168 h), aliquots were taken and centrifuged (1745 xg) for 10 min. pH was determined in the supernatant (Horwitz, 2000) and the precipitate was washed and resuspended with distilled water. Biomass concentration was estimated by measure of absorbance at 620 nm and conversion to dry weight (g/L) using a biomass standard curve previously determined (Kusdiyantini et al., 1998).

Cell disruption techniques

Two different methods for cell disruption were used: chemical disruption with dimethylsulfoxide (DMSO) and enzymatic disruption. The method with DMSO used 0.05 g dry biomass (48 h at 35°C) submitted to freezing (48 h at -18°C) and 2 mL DMSO. The mixture was homogenized by vortexing every 15 min for 1 h (Fonseca et al., 2011).

The enzymatic method used commercial enzyme preparation Glucanex® (Novozymes) from *Trichoderma harzianum*, containing the enzymes β-1,3-glucanase, protease, chitinase and cellulase. A sample (0.011 g dry biomass) was mixed with sodium acetate buffer 0.2 M, pH 4.5 and enzyme extract with initial activity of 0.6 U/mL. The final mixture was incubated at 55°C in an agitated bath for 30 min, centrifuged at 1745 x*g*, and the supernatant was separated for carotenoid extraction (Michelon et al., 2012).

Biomass particle size effect

The biomass obtained at the end of cultivation (168 h) was dried at 35°C for 24 h and frozen for 48 h at -18°C. It was macerated in a mortar and pestle and sieved into different fractions (<32, 32, 42, 80 and 115 mesh) corresponding to particulate sizes of >0.500, 0.500, 0.355, 0.180 and 0.125 mm, respectively. Disruption with DMSO and subsequent extraction with petroleum ether were used to determine the total carotenoids.

Stability of carotenoids during biomass freezing

Dry biomass (0.05 g) was submitted to freezing (-18°C) in flasks with a lid, and compared with the carotenoid content of the unfrozen sample. Disruption with DMSO and subsequent extraction with petroleum ether were used to determine the carotenoids, followed by determination of antioxidant activity.

Extraction and determination of total carotenoids

After the disruption, 6 mL acetone was added to facilitate carotenoid extraction. The sample was centrifuged at 1745 xg for 10 min, then solvent was removed and the disruption procedure was repeated until cells were colorless. The solvent extracts were mixed with 10 mL NaCl 20% (w/v) and 10 mL petroleum ether was added. After stirring and phase separation, the excess water was removed with sodium sulfate (Fonseca et al., 2011). The total carotenoids in the extracts were determined as astaxanthin using a spectrophotometer (Biospectro SP-220, China) at 474 nm (Rodriguez-Amaya, 2001) and the values were defined as in Equation 1, using the specific absorptivity coefficient in petroleum ether (2100 mol/L.cm) (Chumpolkulwong et al., 1997; Domínguez-Bocanegra and Torres-Muñoz, 2004).

Table 1. Gradient elution of the mobile phase.

Time (min)	Acetonitrile (%)	Methanol (%)	Ethyl acetate (%)	Flow (mL/min)
0	70	30	0	1.0
5	80	10	10	0.3
6	30	0	70	1.0
7	30	20	30	1.0
10	70	30	0	1.0
17	70	30	0	1.0

$$C = \frac{A * V * 10^6}{A_{1cm}^{1\%} * 100 * m_{sample}} \quad (1)$$

Where, C = total concentration of carotenoids (µg/g dw); A = absorbance; V = volume (mL); m_{sample} = dry cell mass (g); $A_{1cm}^{1\%}$ = specific absorptivity.

HPLC-VWD analysis of carotenoids

The carotenoids obtained at the end of yeast growth (168 h) without freezing were identified using a high-performance liquid chromatographer (Shimadzu, Kyoto, Japan), consisting of a system of LC-20AT pumps, a DGU 20A5 degasser, a CBM -20A controller, manual gun with 20 µL handle and a SPD-20A spectrophotometric detection system at 450 nm. Instrument control and data acquisition were conducted using LC Solution software. Determinations were made using Discovery Bio Wide Pore C18 Reverse Phase column chromatography, 10 µm (25 cm x 4.6 mm) maintained at room temperature (20°C) using a mobile phase acetonitrile : methanol : ethyl acetate (70:30:0 v/v) in gradient mode (Table 1) with 1 mL/min flow rate and 17 min total run time, and linearity between 0.1 and 7 µg/mL for astaxanthin and β-carotene and between 0.05 and 6 µg/mL for lutein. As chromatographic standards, astaxanthin, lutein and β-carotene were used.

Antioxidant activity of carotenogenic extracts

To determine the antioxidant activity of extracts, these were concentrated in a rotary evaporator at 30°C, and petroleum ether was used as solvent for all the assays. The scavenging of DPPH (2,2-diphenyl-1-picrylhydrazyl) was determined according to the method by Sousa et al. (2007), with modified reaction time. A solution of 5 mM DPPH in methanol was prepared and, protected from light, was mixed with a known quantity of carotenogenic extract. After 60, 120 and 180 min, the absorbance was determined at 515 nm.

The 2,2 '-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) method was applied in accordance with Nenadis et al. (2004), with modified reaction time. A stock solution of 7 mM ABTS was prepared, from which the radical $ABTS^{\bullet+}$ was prepared, and this consisted of reaction between 5 mL stock solution with 88 µL 140 mM potassium persulphate solution. The mixture was protected from light at room temperature for 16 h. Afterwards, it was diluted with ethyl alcohol to obtain an absorbance of 0.70 ± 0.05 at 734 nm. In the dark, the radical $ABTS^{\bullet+}$ was added to test tubes along with carotenogenic extracts to complete 4 mL in each tube. The reaction was monitored every 15 min at 734 nm.

The reduction power of iron (ferric reducing antioxidant power – FRAP Assay) was determined based on the protocol developed by Benzie and Strain (1996). The FRAP reagent was prepared from a solution of 0.1 M acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM ferric chloride (10:1:1 v/v/v) (Chang et al., 2007). The reagent was heated to 37°C and the carotenoid extract was added at the end of this time. The reduction of Fe(III)-TPTZ was monitored every 15 min by absorbance reading at 593 nm.

6-Hydroxy-2,5,7,8-tetramethylchloromane-2-carboxylic acid (Trolox; Sigma-Aldrich Chemical) was used to construct calibration curves and all the results are expressed as Trolox equivalents (in mM of Trolox per µg of sample).

Statistical analysis

Results were submitted through analysis of variance (ANOVA). The mean values were compared by Tukey's test at a 5% significance level using the Statistica software (version 5.0, StatSoft, Inc., USA).

RESULTS AND DISCUSSION

Influence of particle size and freezing time of biomass on carotenoid extraction using cell disruption with DMSO

Biomass of *P. rhodozyma* NRRL-Y 17268 obtained at 168 h cultivation was 4.4 ± 0.4 g-dw/L, final pH was 8.4 ± 0.1 and, after disruption with DMSO and extraction, the content of total carotenoids in dried biomass was 215.0 ± 4.4 µg/g-dw, prior to standardization of the particle size of the biomass of the yeast.

The extraction of carotenoids was facilitated by decreasing the particle size, which can be shown through statistical analysis (Table 2). The total carotenoids content extracted from the biomass and collected in sieves opening 115 mesh was highest (264 µg/g-dw) and significantly different from the others, probably due to the cell/solvent contact area. Biomass with a larger particle size (> 0.500 mm) resulted in lower recovery of carotenoids, while in the case of intermediate sizes (between 0.500 and 0.180 mm), there was no significant difference between them. Therefore, biomass particle was established as 0.125 mm in order to obtain a higher yield of carotenoids. The particle sizes considered in this work are the average of the particles that pass through the sieve.

The method using DMSO is the cell disruption process

Table 2. Total carotenoids extracted from biomass of *P. rhodozyma* NRRL-Y 17268 with different particle sizes using cell disruption with DMSO.

Mesh	Aperture diameter of sieves (mm)	Total carotenoids (µg/g-dw)*
>32	> 0.500	26.0 ± 4.5[c]
32	0.500	192.0 ± 9.9[b]
42	0.355	204.0 ± 0.2[b]
80	0.180	215.0 ± 10.4[b]
115	0.125	264.0 ± 3.9[a]

*Mean values ± standard deviation for n determinations (n=3). Different letters in the same column indicate a significant difference (p <0.05).

Table 3. Total carotenoids from *P. rhodozyma* NRRL-Y 17268 biomass (0.125 mm) subjected to freezing (-18ºC) for increasing periods of time, using cell disruption with DMSO.

Freezing time (h)	Total carotenoids (µg/g-dw)*
0	92.0± 3.6[c]
48	272.0 ± 8.1[a]
120	265.0 ± 5.1[a]
192	251.0 ± 2.9[a]
264	176.0 ± 2.4[b]
336	170.0 ± 3.2[b]
408	144.0 ± 9.5[b,c]
480	125.0± 5.7[c,d]
552	102.0± 5.9[c,d,e]
624	92.0 ± 3.9[d,e]
720	80.0 ±3.56[e]

*Mean ± standard deviation for n determinations (n=3). Different letters in the same column indicate a significant difference (p <0.05).

most widely used for the extraction of total carotenoids from *P. rhodozyma* biomass as it promotes a change in composition and permeability of membranes (Michelon et al., 2012). This method proves to be more efficient for total carotenoids recovery. However, due to its high toxicity, DMSO is usually used for quantification on an analytical scale, as in the case of this study.

The results of freezing at different times on the recovery of carotenoids are shown in Table 3. The content of total carotenoids extracted from the frozen biomass for 48 h is three times higher and significantly different when compared with biomass without freezing. This confirms that the freezing procedure was indeed favorable and facilitates the extraction of carotenoids. However, with the increase in freezing time, the recovery of carotenoids decreased (at 264 h, a significant difference was observed in comparison with the observations made after 192 h), revealing a negative effect on the extraction process, with a gradual decrease until 720 h. Between 48 and 192 h of freezing, no significant differences were observed. Therefore, it was not necessary to freeze the biomass for more than 48 h.

Several authors have studied the influence of storage temperatures on carotenoids from fruits and vegetables. Leong and Oey (2012) studied the effects of processing (98°C for 10 min) and freezing (-20°C in liquid nitrogen), as well as drying and freezing, on the contents of anthocyanins, carotenoids and vitamin C from cherries, nectarines, apricots, peaches, plums, carrots and red peppers. They found that freezing induced the formation of ice crystals favoring extraction and reallocation of molecules and water within cell structure. They also showed that when cultivars were submitted to freezing, most had an increased content of carotenoid, as found in this study, although a different freezing method was used. However, these authors did not evaluate the antioxidant activity of carotenoids.

Influence of freezing time of biomass on antioxidant activity of carotenogenic extracts obtained by cell disruption with DMSO

Carotenogenic extracts obtained from different biomass freezing intervals (0, 24, 48 and 72 h) were evaluated in relation to the ability to inhibit DPPH radical, ABTS radical and reduction power of iron (FRAP), in order to measure antioxidant activity. The methods for the determination of antioxidant activity were expressed in mM trolox/µg of carotenoids (Table 4), which relates the concentration of carotenoids in each extract and the maximum time of reaction for each method.

The carotenoids obtained from biomass without freezing had significantly higher antioxidant activity than carotenoids extracted from frozen biomass in all the methods under study, with 0.77, 2.05 and 4.30 mM trolox/µg for ABTS, FRAP and DPPH (Table 4), respectively.

In general, the antioxidant activity presented in Table 3 decreased with increased freezing time for all methods in question. The method that suffered the greatest decline, 4.30 to 0.96 mM trolox/µg, was the DPPH. The FRAP method also present a decline with the freezing time in

Table 4. Antioxidant activity (mM trolox/µg) for the carotonogenic extracts of *P. rhodozyma* NRRL-Y 17268.

Freezing time (h)	DPPH	ABTS	FRAP
0	4.30 ± 0.05^{aA}	0.77 ± 0.03^{aC}	2.05 ± 0.24^{aB}
24	0.96 ± 0.09^{bcB}	0.75 ± 0.11^{aB}	1.62 ± 0.05^{bA}
48	0.99 ± 0.03^{bA}	0.37 ± 0.01^{bB}	0.99 ± 0.09^{cA}
72	0.84 ± 0.00^{cB}	0.72 ± 0.04^{aB}	1.45 ± 0.05^{bA}

Mean values ± standard deviation (n=3). Different lowercase letters in the same column indicate significant difference. Different uppercase letters in the same row indicate significant difference ($p<0.05$).

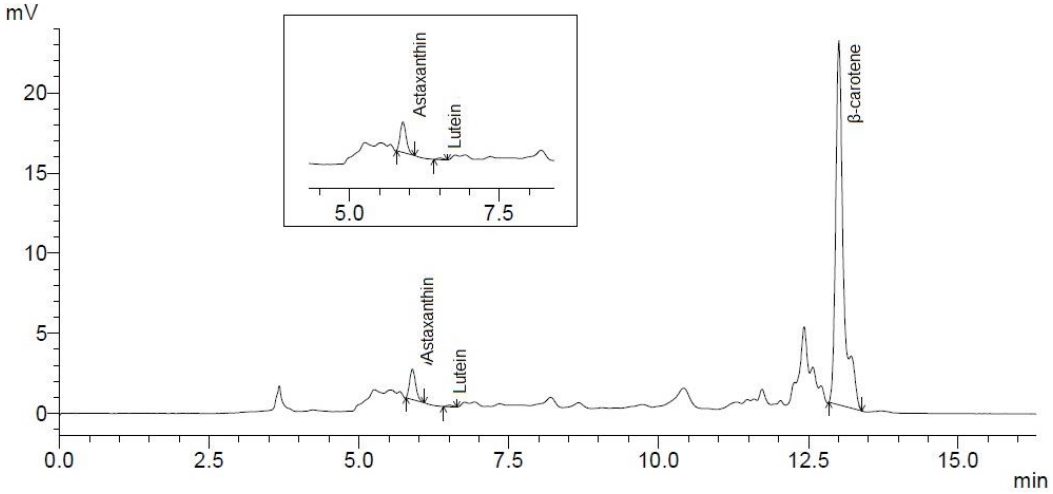

Figure 1. Chromatogram of carotenoids obtained from carotenogenic extract from *P. rhodozyma* NRRL-Y 17268 using cell disruption with DMSO without freezing. SPD-20A Spectrophotometric detection system 450 nm. Reversed Phase Chromatography Discovery Bio Wide Pore C18, 10 µm (25 cm x 4.6 mm).

the first 24 h, from 2.05 to 1.62 mM trolox/µg, while for the ABTS method, the values at 0 and 24 h, there was no significant difference.

Considering that the ice expands, disrupting the cell wall and potentially making carotenoids more accessible to solvents, increasing recovery, the antioxidant activity would also be expected to increase with the growing concentration of carotenoids. However, such behavior was not observed in this study.

Figure 1 shows the chromatogram of carotenoids obtained from carotenogenic extracts of *P. rhodozyma* NRRL-Y 17268 using cell disruption with DMSO without freezing. It was possible to verify the presence of β-carotene, lutein and astaxanthin, while 81% of the carotenoids identified correspond to the first. These are indicated as singlet oxygen binders, and this is related to a conjugated double bond system, which is likely to have resulted in the highest antioxidant activity without freezing.

The three carotenoids cited have nine double bonds in the main chain, which confer greater activity (Rodriguez-Amaya, 2001). Thus, with the freezing, there may have been a release of some compounds during this process, which may have inhibited the activity or have affected the determination of antioxidant potential. The degradation of these carotenoids may also have occurred. Another hypothesis for the decreased antioxidant activity throughout the freezing is the formation of cis isomers, which have lower biological activity (Rodriguez-Amaya et al., 2008). Furthermore, carotenoids may have been oxidized by exposure to air (Britton, 1995) in such a way as not to have a high antioxidant activity, although freezing may have promoted a more efficient cell wall disruption, reflected in higher levels of pigment recovered (Table 3).

The values of antioxidant activity of the carotenogenic extracts depend on the method employed and the conditions of the reaction. Bolanho et al. (2014) studied formulations of cookies enriched with *Spirulina platensis* (which contains high level of total phenolic compounds).

They obtained antioxidant activities ranging from 31.1 to 36.5 mmol trolox/kg with the DPPH method, 0.7 to 1.1 mmol trolox/kg with the ABTS method, and 12.1 to 17.0 with the FRAP method. Also, in terms of comparison values, Hayes et al. (2011) analyzed the antioxidant capacity of four commercial phytochemical products (olive leaf extract, lutein, sesamol and ellagic acid). The best results were for ellagic acid with 1.97 and 1.70 mmol trolox/100 g DW for the ABTS and FRAP, respectively.

Due to its structure, the carotenoids can interact differently in each methodology of antioxidant activity, so it is necessary to use several methods, which help to elucidate its mechanism of action (Parrila et al., 2005; Fu et al., 2011). The proposed methods had differences and similarities, resulting in different behaviors in relation to interaction with microbial carotenoids. The DPPH radical decreased over biomass freezing time, while ABTS and FRAP decreased until 48 h.

Besides the yeasts, other microorganisms are capable of producing carotenoids with antioxidant potential. Goiris et al. (2012) studied the antioxidant potential of micro-algae in relation to its content of phenolic compounds and carotenoids, using ABTS, FRAP and AIOLA (a method that measures the ability of the antioxidant to prevent oxidation of linolenic acid), obtaining maximum values of 48.9, 89.7 and 46.3 µmol trolox eq/g for *Phaeodactylum tricornutum*, stating that the carotenoids present in the extracts are mainly responsible for the antioxidant activity of this microalga.

Antioxidant activity of carotenogenic extracts from *P. rhodozyma* NRRL-Y 17268 obtained by cell wall disruption by enzymatic method

Since the aim is to obtain an extract with potential application in foods, enzymatic lysis of the cell wall would be ideal because it does not present the possible risks of toxicity shown by chemicals. Thus the DDPH, ABTS and FRAP antioxidant activity methods were conducted with the extract obtained from the enzymatic rupture of *P. rhodozyma* cells.

Although the freezing led to a greater recovery of carotenoids from *P. rhodozyma* NRRL-Y 17268 (Table 3), a negative influence was observed concerning anti-oxidant activity with increasing freezing time (Table 4). Therefore, biomass was used without freezing in the enzymatic lysis procedure.

The cell disruption method for biomass without freezing was applied using commercial enzyme preparation Glucanex®, obtaining total carotenoids of 267.3 ± 0.6 µg/g, statistically equal to values obtained with DMSO; 264.0 ± 3.9 µg/g (Table 2), according to test t ($p<0.05$).

The interaction of carotenoids obtained from enzymatic cell disruption with the DPPH radical was not observed. This non-detection may be associated with the release of secondary cell wall compounds or the release of the enzymatic preparation itself, which also has the enzymes protease, cellulase and chitinase. Moreover, interaction between microbial carotenoids and the ABTS radical was observed (Table 5), achieving values of 4 mM trolox/µg in 75 min, which are higher than those found previously using chemical disruption with DMSO. This increase was not observed in the extracts obtained by chemical disruption with DMSO (data not show).

Table 5. Antioxidant activity (mM trolox/µg) for the carotonogenic extracts of *P. rhodozyma* NRRL-Y 17268 using an enzymatic method of cell disruption.

DPPH (min)	ABTS (min)	FRAP (min)
ND (0 min)	2.42 ± 0.03 (15)	2.16 ± 0.04 (15)
ND (120 min)	2.71 ± 0.07 (30)	2.24 ± 0.03 (30)
ND (180 min)	2.89 ± 0.05 (45)	2.24 ± 0.04 (45)
-	3.49 ± 0.05 (60)	3.00 ± 0.05 (60)
-	4.00 ± 0.01 (75)	3.66 ± 0.11 (75)

Mean ± standard deviation. (n=3). ND, not detected. Different lowercase letters in the same column indicate significant difference. Different uppercase letters in the same row indicate significant difference ($p<0.05$).

The method using the enzymatic disruption was milder and more ideal for cell disruption of *P. rhodozyma* NRRL-Y 17268. Although the antioxidant activity of carotenogenic extract was not detected by the method using the free radical DPPH, the other methods showed a significant improvement, suggesting that this method of cell disruption affects favorably antioxidant activity.

Therefore, through the use of enzymatic cell wall disruption, the highest values of antioxidant activity of this study were obtained for both the ABTS and FRAP methods: 4.00 and 3.66 mM trolox/µg, respectively.

Conclusions

This study aimed to explore two different disruption methods in relation to the antioxidant activity. Although the use of freezing favored the extraction of carotenoids, the carotenoids extracted not necessarily had higher antioxidant activity. Using the enzymatic method of disruption of the cell wall, it was possible to obtain the highest values of antioxidant activity, using the ABTS and FRAP methods, with results of 4.00 and 3.66 mM trolox/µg, respectively. The extent of this activity was shown to be influenced by three different methods: the DPPH, ABTS and FRAP. There is still much to be explored in this field, mainly because of the importance of this study, and the future application of microbial carotenoids obtained by submerged cultivation is promising. Therefore, this study has contributed to the use of microbial carotenoids as a source of biologically active compounds, also contributing to future studies in this field.

Conflict of interests

The author(s) did not declare any conflict of interest.

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