African Journal of Biotechnology Vol. 9(19), pp. 2811-2815, 10 May, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

A semi-automatic system for labelling seafood products and obtaining fishery management data: A case study of the bottom trawl fishery in the central Mediterranean Sea

Gioacchino Bono*, Salvatore Cusumano, Cinzia Badalucco, Vito Pipitone and Sergio Vitale

Istituto per l'Ambiente Marino Costiero, Consiglio Nazionale delle Ricerche, Via L. Vaccara, 61, 91026 Mazara del Vallo, Italy.

Accepted 16 April, 2010

This study is on the implementation of a semi-automatic labelling system (LS) of the Mediterranean Sea seafood harvest to address the increased need for seafood authentication and inherent difficulties of commonly used indirect techniques for estimating fisheries yield and fishing effort. Sensitive data required for anti-counterfeit policies, such as date and catch area, can be acquired and recorded on the label by user-friendly automated software that excludes any possible manipulation by the crew. Based on results obtained from the installation of the LS on bottom commercial trawlers, the system certified the origin of the seafood products and simultaneously provided, indirectly, geospatial fisheries yield and fishing effort data of the main exploited species.

Key words: Seafood, labelling, Mediterranean Sea, data management.

INTRODUCTION

Market integration has become, in recent decades, the most significant step in the evolution of market economies. The wide use of information communication technology (ICT) systems and a significant reduction in merchandise transportation costs are bringing international markets increasingly closer. This phenomenon, which has become well known under the term globalization, has reached every economical sector to varying degrees.

Based on a general tendency towards globalization of markets, the price of various production processes are progressively declined, striving (as can be expected) for the lowest production costs. The lowest costs do not necessarily correlate to higher productivity and efficiency, but are simply associated with lower production costs and different workforce rules of engagement. The response to this general trend, especially in developed economies, has been product differentiation and valuation of local specialty products. Strategies such as these require tools geared toward reducing the asymmetric information between the producer and consumer. Without efficient informative tools that allow identification of the diverse quality of products, as underlined by Akerlof (1970), consumers run the risk of purchasing goods that in reality

*Corresponding author. E-mail: gioacchino.bono@cnr.it. Tel: +39 0923 948966. Fax: +39 0923 906634.

Abbreviations: LS, Labelling system; **ICT,** information communication technology; **GPS,** global positioning system; **GSA,** geographical sub-area; **GFCM,** general fisheries commission for the Mediterranean; **FAO,** food and agriculture organization; **NMEA,** national marine electronics association; **CNR,** Consiglio Nazionale delle Ricerche; **GIS,** geographical information system; **EAN,** European article numbering; **RFID,** radio frequency identification; **VMS**, vessel monitoring systems.

they do not want. This reduces the consumer's willingness to pay, which in turn forces better products to leave the market or to use a single market (at a single price). The market for fish products is not isolated from the globalization process and the Mediterranean sector in particular is the focus of our study.

The Mediterranean fish industry is of special interest since the general effects of globalization are compounding with the inefficiency of traditional strategies to protect the shared fishery resources. The presence of 24 countries around a relatively closed basin (each with a different culture, legal tradition and levels of economic development) have in fact restricted the adoption of any method of resource regulation, thereby contributing to an increasing depletion of resources. In addition, reduced fish stocks have increased the resource extraction costs (that is, increased fishing effort is required), thereby trapping Mediterranean producers in an economical tradeoff; on one side is the need to adapt to decreasing international prices and on the other the need to absorb increasing local production costs. The current economic policy strategy adopted to meet this double pressure on the fishing industry has been to subsidize the various sector enterprises by many governments, thereby transferring the losses from the single operator to the collective whole without directly addressing the problem of competition by Mediterranean producers and the problem of sustainable resource management.

Far from attempting to offer the panacea to solve all problems, the system we propose attempted to contribute to the solution, from a methodological point of view, by addressing two specific problems that are considered to be prominent topics in the seafood industry: (1) To enable accurate information transfer between producers and consumers; and (2) to facilitate data collection, which is essential for fish stock assessment. To address these objectives, we developed a labelling system tailored to Mediterranean production systems currently in place. The system represents an important informative tool for identifying the origin of products and is the foundation for an efficient tracking (Borresen and Frederiksen, 2004) system that accompanies the data through the entire market channel down to the end consumer. Furthermore, this identification system allowed for the adoption of concrete product differentiation strategies and evaluation of Mediterranean local specialty products. At the same time, the labelling system offers the opportunity to collect geospatial fisheries yield and fishing effort data for the main exploited species. This information would help better address some of the problems facing traditional fisheries management actions that were inadequate to safeguard the decline of fish stocks (Pauly et al., 2002).

In particular, in the central Mediterranean sea, some indirect acquisition methods of fishery-dependent data based on auto declaration by the producers and/or on landing interviews (Bazigos et al., 1984, Andreoli et al., 2000; Spagnolo and Placenti, 1998) are not reliable since the data are provided several days after fishing operations and therefore, the data are reconstructed accounts and are not tied to real-time observations.

Described below is the structural and functional architecture of a system designed for the trawl fishing industry and a description of its strengths, which may contribute to an increased flow of information between producers and consumers and give authenticity to the essential data needed to evaluate commercial fish stock in the Mediterranean.

MATERIALS AND METHODS

The labelling system is based on years of direct and careful observation of all the phases of the Mediterranean bottom trawl fishery including the packaging and stacking of fish products until the vessel reaches the harbor. The planning of the LS considered both the lack of familiarity of fishermen with ICT instrumentation and the inherent difficulties involved when attempting to modify well-coordinated work patterns. For these reasons, the hardware components and software development have been carefully designed with solutions geared towards minimizing system-operator interaction and maximizing the simplicity of procedures to be done on board.

System description

The functional and architectural LS schematics (Figure 1) can be described considering: weighing and labelling subsystem (WLS); database and transmission management subsystem (DTMS); database and web server (DWS) which is an optional system for data analysis or for real-time commercialization activities.

WLS

The components of this subsystem include a marine scale, processing-acquisition hardware and a label printer. These components are installed in a damp area of the fishing vessel where fish are normally processed and therefore, must contain water-protection mechanisms to prevent water infiltration and subsequent corrosion induced by salt water.

The processing-acquisition unit utilizes a touch screen interface and the management system was developed to minimize the role of the operator. From a functional point of view, the new system works in the following manner:

(1) The weighing operation is allowed only after the end of a haul for which the captain (through the DTMS software subsystem) has given permission (haul authorized weighing); otherwise, it disables label printing.

(2) In the processing area, the operator can video-select the fish species (that for simplicity are presented with an icon or commercial name), the size-grade (first, second, third, etc.), the type of processing

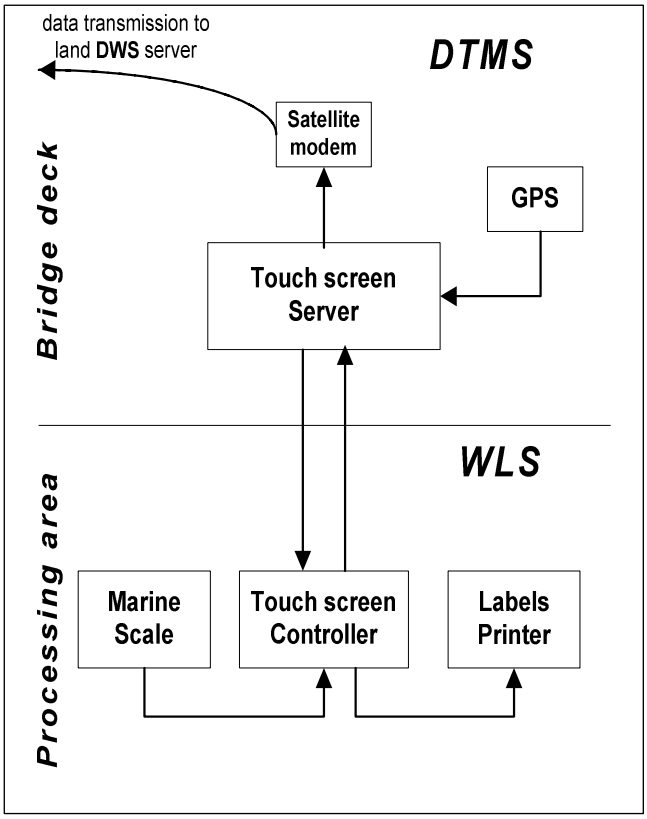

Figure 1. The fishing vessel operations and data flow. Video of the system is available: http://lnx.utp-iamc.org/index.php?option=com_content&task=view&id=22&Itemid=29.

(whole, head-off, gutted, etc.), and the type of packaging used (tray, box, etc.).
(3) The operator, through a simple push of a button or the touch of a screen area, activates the self-adhesive label printing that can then be attached to the package.

This process reduced the number of steps that must be completed by the operator, especially since the weighing operation is normally done by one commercial category at a time (for example, all shrimp matching one size are processed, then another size or species, etc.). Therefore, the steps required are limited only to positioning the box on the scale and to pressing a single button (to print the label; operation is described below in section 3).

All other data printed on the label (including scientific name, date, fishing vessel, company owning ship, catch area, setting number, fish type, etc.) are obtained automatically by the DTMS positioned in the bridge deck.
The subsystems connections (WLS and DTMS) can be done with a single UTP cable, through a small LAN Local Area Networks), or wirelessly. To avoid improper use by on-board personnel that are not authorized to execute weighing and labelling operations, both subsystems WLS and DTMS require a username and password during the access phase.

DTMS

This subsystem located in the bridge deck, is connected to the global positioning system (GPS) and collects in its database all data relevant to ship vessel activity. The following statics data is inserted only once during the system configuration: ship vessel identification data (name, registration number), identification of the company that owns the ship, the scientific and common name of species captured, whether they be fish, crustaceans or cephalopods, the type of production, etc. In a specific table, all dynamic data are saved for each catch, according to the following fields: (1) Haul number; (2) catch area; (3) species name; (4) size/grade; (5) weight; (6) latitude at start of haul; (7) longitude at start of haul; (8) date and hour at the start of haul; (9) latitude at the end of haul; (10) longitude at the end of haul; (11) date and time at the end of haul; (12) catch area according to allocation in macro-areas and geographical sub-area (GSA) pursuant to general fisheries commission for the Mediterranean (GFCM, 2000). More specifically, the DTMS carries out the following functions:

(1) Obtains in an entirely automated manner the geographical location of the fishing vessel through a GPS positioning device;
(2) Allows the Captain to determine the start and stop of haul and to activate the weighing and labelling operations;
(3) Memorizes all the fishing activity data from both DTMS and WLS;
(4) Automatically sends the capture data to DWS.

Identification of the capture zone in accordance with food and agriculture organization (FAO)-GFCM requisites is done automatically through the execution of a proper algorithm (Area Finder) developed in the DTMS software that acquires the geographical coordinates from the GPS according to National Marine Electronics Association (NMEA) standards.

DTMS also allows transmission to land of the catch data obtained over the course of the day. A software module was programmed to generate a text file every 24 h with the daily catch data. Therefore, a file is created for every fishing day; this file is then added to the previous records in the same folder. DTMS by a scheduler transmits data daily via a satellite modem connected to the internet to access a file transfer protocol (FTP) server. All settings and authentication credentials for server connection (telephone number of the internet provider, username and password for internet and FTP access) are pre-set on the DTMS software settings. The DTMS software transmission module allows for appropriate adjustments to synchronize data and troubleshoot connection problems (connection interruption, satellite unavailability, etc.) The connection method and the use of small daily log files for catch data transfer have been thoroughly designed to minimize transmission costs. In fact, a real-time transfer (technically possible and doable) of catch data to a land server via satellite has been discarded due to costs associated with this solution.

DWS

This is an optional component that is non-essential for fish labelling. In the LS testing phase, this server was installed on-site at the Consiglio Nazionale delle Ricerche (CNR) to carry out essential functions:

(1) To receive and process data from fishing vessel via satellite

communication;
(2) To allow equal access to the vessel owners in the project via a Web server.

As previously stated, it is possible to obtain the catch data via the internet and an FTP client installed in the machine. For the FTP Server, a freeware software has been used (ServU-32), configured in a way to minimize the risk of a hacking attack. The files received are stored in an adjoining directory that is later imported, through a specially designed application, into a database table. This application is also done daily through the system scheduler. The scheduler application execution time is postponed by one hour after transmission to ensure there is no interference with the fishing vessel transmission.

The application functions in a batch mode and conceptually performs the following operations: (1) Checks the work file contents; (2) verifies that files found have already been imported; (3) adds the new file records received to the database table. The database application contains, in addition to the "service tables" ("species" and "imported files"), the records of users who could have had access to the data via a Web server.

Once the data is received by the fishing vessel and have been imported to the database, they are immediately available via a Web server. Data obtained through the DWS may be viewed in a summarized form and are only accessible by authorized users (typically the vessel-owners and their staff). The catch data may be itemized based on the following criteria: (1) Period or date; (2) Single commercial species; (3) All species.

RESULTS AND DISCUSSION

This labelling system has been tested by three fishing vessels equipped with different hardware systems and scales marketed by three industry leaders in the European region. All configurations installed gave positive results and met project expectations. Below are a few of the LS system strengths:

(1) It was a good replacement of the current fish labelling system, which is based on manual and non-transparent data entry of pre-print labels.
(2) It was flexible and complementary to the established consolidated operative actions normally done by fishermen. The system, in fact, was designed to be as user-friendly as possible, by minimizing the number of steps and making them simple and intuitive.
(3) In compliance with European law (article 4, regulation 104/2000 and article B of regulation 2065/2001), the fish labels contained the required minimum information components, and were enhanced with additional elements that provided precise and qualifying information to consumers, such as the catch area, for example. In fact, to comply with existing standards, only one of the three FAO macro areas (ex. 37.1, 37.2 and 37.3) where the product was caught is required, but this system also added the GSA where the vessel was located at the time the product was caught and labeled. An improvement was therefore made towards greater geographical characterization of fishery products.
(4) Data reliability was improved; the information printed on the label did not require subjective entries entered by the shift worker, but were instead obtained by automatic detection equipment.
(5) Relative affordability and quality of the entire system, including both hardware and software. In regards to the software, the development of the "Area Finder" module allowed the replacement of an expensive license to use the GIS (geographical information system).
(6) The system is the starting point for the development of an application capable of tracking fish products from "sea to dish" (Frederiksen et al., 2002; Thompson et al., 2005). Rapid advances have been made in IT standard methods used to code any consumer good, such as European article numbering (EAN) or, even better, Radio Frequency Identification (RFID) tags. These systems may use the information obtained to build the basic elements of a successful seafood tracking program.
(7) The LS did not only address the needs tied to fish labelling but, as previously stated, was designed to facilitate assessment of the dynamics of exploited fish populations over time (Hilborn and Walters, 1992). In fact, the collective action of the captain who works on the DTMS (registers the haul and fishing time) and the operator working on the WLS (registers the catch composition), produced a string of time-space fishing effort and yield fisheries data. These data are the fundamental tools to the most common fish stock assessment methodologies. Furthermore, the commercial fishing vessels became useful Vessel Monitoring Systems (VMS) that could extend to a significant group of vessels, over a specified fishing ground, which would increase the chances of success in the management and control of Mediterranean fishing.
(8) This system is implementable in all bottom trawl fishing vessels larger than 12 m. Thus, in alternative (or in complementary) to traditional paper logbook, it stands as an accurate catch reporting tool to the improvement of the national statistical and information system, targeting the Mediterranean fishery (Coppola, 2007; GFCM, 2009).
(9) Lastly, such information would integrate with other direct methods of data collection carried out by the various trans-national research programs based on experimental trawl surveys (Abellò et al., 2002) carried out by the European countries.

After testing this system, it was clear that transparent labelling in the Mediterranean fishing industry is possible and convenient. The effectiveness of this system was closely connected to a user-friendly architecture integrated (in an automated format) with all the information normally found on board. It was also the only instrument available

to enable Mediterranean product diversification on global markets and for social responsibility towards the shared resources.

The increased transparency between the producer and consumer provided a method to identify the Mediterranean fish product in an accurate and unmistakable manner, giving specific responsibilities and subsequent economic benefits, to the fishing vessel. These benefits included incentive not to ignore the entire product distribution chain, acquire and maintain credibility, reduce the substitution rate of products in the market and obtain a greater rate of return. These responsibilities fostered consumer choice and the consumer in turn regained the tools needed to reward high quality and increased product transparency.

The advantages of the labelling system studied extend, based on our opinion, beyond the important flow of information from producers and consumers. Assuming that the system can be extended to a greater number of vessels, the information obtained (catch zone, time, species and quantity) could be statistically processed and returned in aggregate form to the operator, allowing the best measurement of the quantity withdrawn, the fishing effort and estimates of the profit. Since a system such as this could be progressively extended across the Mediterranean region, it could become a regional database. This has a double benefit for fishing operators: prevention of foreign products to enter in the market passing as Mediterranean products and easy management of the production levels that reach the market. Verification means being able to address both demand segmentation and price differentiation and also to obtain increased knowledge of exploitation levels and the catch-effort relationship.

The knowledge of self-action is a central element in the efficient use of collective resources, since it tends to internalize the negative externality impact of resources exploitation. With that aim in mind, an awareness is not only theoretical on the negative impact of each persons actions on the collective resource, but also concrete knowledge (measured with the database provided by the operators themselves) of the effects that fishing has on species conservation could form the base for a new model of resource management. A model not necessarily imposed by traditional regulatory systems but that monitors resources with a new sensitivity and social responsibility such an approach well regarded by the recent Nobel Prize recipient in Economics, Elinor Ostrom (1990).

REFERENCES

Abellò P, Bertrand JA, Gil de Sola L, Papaconstantinou C, Relini G, Souplet A (2002). Mediterranean Marine Demersal Resources. The Medits International trawl survey (1994-1999). Scientia Marina. 66(2): p. 280.

Akerlof G (1970). The Market for Lemons: Quality Uncertainty and the Market Mechanism, Quarterly J. Econ. 84: 485-500.

Andreoli MG, Levi D, Cannizzaro L, Garofalo G, Sinacori G (2000). Sampling statistics of southern Sicily coastal trawl fisheries: Minipestat programm. SAC-GFCM-Sub-Committee of Stat. Info. Madrid. Spagna. p. 18.

Bazigos GP, Cingolani N, Coppola SR, Levi D, Mortera J, Bombace G (1984). Studio di fattibilità per un sistema di rilevazione campionaria delle statistiche della pesca (PESTAT). ParteI: Statistiche sulla flottiglia da pesca. Quaderni dell'Istituto Ricerche Pesca Marittima. 4(1): p. 16, 358.

Borresen T, Frederiksen M (2004). Technical possibilites for introducing traceability in the seafood industry. In: Shahidi F, Simpson BK editors. Publishing Company, St John's, NL, Canada. Seafood quality and safety. Sci. Technol. pp. 31-38.

Coppola SR (2007). An adaptive approach for the improvement of fishery statistical systems in Mediterranean countries under FAO projects. No. 79. Rome, FAO. Studies and Reviews. General Fisheries Commission for the Mediterranean. p. 54.

Frederiksen M, Osterberg C, Silberg S, Larsen E, Bremner A (2002). Info-Fisk. Development and validation of an internet based traceability system in a Danish domestic fish chain. J. Aquat. Food Prod. Technol. 11(2): 13-34.

GFCM (2000). Report of the 2nd Session of the Sub Committee on Stock Assessment. Madrid (Spain), 26-28 April 2000. General Fishery Commission for the Mediterranean. Scientific Advisory Committee. Sub-Commun. on Stock Asses.

GFCM (2009). Report of the Workshop on the GFCM Regional Logbook for the Mediterranean and Black Sea. Rome, Italy, 29 June -1 July 2009. General Fishery Commission for the Mediterranean. Scientific Advisory Committee, Sub-Committee on Stat. Info.

Hilborn R, Walters CJ (1992). Quantitative Fisheries Stock Assessment: Choice, Dynamics and Uncertainty. Chapman and Hall, New York. p. 570.

Ostrom E (1990). Governing the commons. The Evolution of Institutions for Collective Action. Cambridge University Press. p. 280.

Pauly D, Christensen V, Guenette S, Pitcher TJ, Sumaila UR, Walters CJ, Watson R, Zeller D (2002). Towards sustainability in world fisheries. Nature,. 418: 689-695.

Spagnolo M, Placenti V (1998). I sistemi di informazione statistica della pesca in Italia. Quaderni I, Angeli F Ed. p. 157.

Thompson M, Sylvia G, Morrissey MT (2005). Seafood traceability in the United States: Current trends, System design and potential application. Compr. Rev. Food Sci. Food Saf. 1: 1-7.

African Journal of Biotechnology Vol. 9 (19), pp. 2816-2820, 10 May, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Chitosan activates defense responses and triterpenoid production in cell suspension cultures of *Betula platyphylla* Suk.

Guizhi Fan, Xiaocan Li, Xiaodong Wang, Qiaoli Zhai and Yaguang Zhan*

College of Life Sciences, Northeast Forestry University, Harbin 150040, China.

Accepted 5 April, 2010

Triterpenoid production and plant defense enzyme activity in suspension cultures of *Betula platyphylla* Suk. cells treated with chitosan elicitor were investigated. Chitosan, at the optimal concentration of 100 mgL^{-1} enhanced triterpenoid production by 2.6-fold, with a maximum yield of 202.75 versus 78.72 mgL^{-1} of the untreated control cells. Furthermore, the activities of superoxide dismutase, phenylalanine ammonialyase, endochitinases and exochitinases increased by 1.6 ~ 3.0 fold. These results indicate that triterpenoid accumulation in *B. platyphylla* Suk. cell are the consequence of a plant defense response to chitosan elicitation.

Key words: Chitosan, *Betula platyphylla* Suk., defense enzymes, triterpenoids.

INTRODUCTION

Triterpenoids, extracted from the bark of *Betula platyphylla* Suk., are an excellent drug for its antiviral, antibacterial, antitumor, anti-aids properties (Jing et al., 2005; Alakurtti et al., 2006; Wen et al., 2007). Due to the slow-growth of *B. platyphylla* Suk. trees in nature, *B. platyphylla* Suk. triterpenoids supply has to resort to alternative sources rather than the natural plants. In a previous study, we found that triterpenoids (betulin and oleanolic acid) may be produced in *B. platyphylla* Suk. cell culture, but their content was significantly lower than from the bark of *B. platyphylla* Suk. (Fan et al., 2009a). Therefore, enormous efforts have been made in the search and development of plant cell culture techniques for efficient production of triterpenoids (Fan et al., 2009b; Wang et al., 2008a; Wang et al., 2008b).

Chitosan (β-1,4-linked glucosamine) is a deacetylated derivative easily obtainable from various sources, particularly from the exoskeletons of crustaceans. It is also found in cuticles of insects as well as in the cell walls of fungi and some algae (Sanford and Hutchings, 1987). Being present in the wall of pathogenic microorganisms, it can be recognized as a microbe associated molecular pattern (MAMP) by the plant immune system, thus activating plant defense responses (Iriti and Faoro, 2009) and the related biosynthesis of secondary metabolites. Therefore, chitosan has been widely applied as a potent elicitor in plant cell suspension cultures to enhance secondary metabolite production, such as menthol (Chang et al., 1998), plumbagin (Nahálková et al., 1998), paclitaxel (Zhang et al., 2000), artemisinin (Putalun et al., 2007), phenylethanoid glycosides (Liu et al., 2008) and phenylpropanoid derivatives (Chakraborty et al., 2009). However, the study of Sánchez-Sampedro and co-workers showed that different concentrations of chitosan (5 ~ 200 gL^{-1} culture medium) did not stimulate any increase in silymarin accumulation in *Sylibum marianum* cells (Sánchez-Sampedro et al., 2005). Also, Eilert et al. (1984) found that addition of chitosan resulted in reduction in cell growth and artemisinin content in cultures of *Ruta graveolens*. From the above results it can be concluded that the function of chitosan as elicitor is species specific.

The objective of the current work was to verify the eliciting capacity of chitosan in improving plant growth

*Corresponding author. E-mail: aguangzhan@126.com. Tel: 0451-82191752.

Abbreviations: PAL, Phenylalanine ammonia lyase; **NBT,** nitro blue tetrazolium reaction; **MAMP,** microbe associated molecular pattern; **SOD,** superoxide dismutase; **ROS,** reactive oxygen species; **PR,** pathogenesis-related; **EDC,** endochitinase; **EOC,** exochitinase.

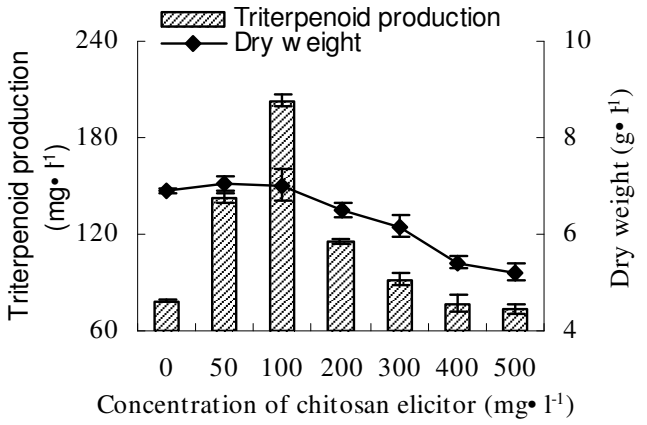

Figure 1. Cell growth and triterpenoid production following chitosan elicitation. Chitosan was added to 8-day- old *Betula platyphylla* Suk. cell suspension cultures, that were harvested after five days of incubation. Control cultures were added of the same amounts of distilled water. Vertical bar represents standard error of three replications.

and triterpenoid accumulation in *B. platyphylla* Suk. cell suspension cultures and to correlate this possible enhanced accumulation with the activation of plant general defence responses, that is, the activities of superoxide dismutase (SOD), phenylalanine ammonia-lyase (PAL), endochitinases (EDC) and exochitinases (EOC).

MATERIALS AND METHODS

Plant materials

The cell line used in the present study was developed from the axillary buds of 30-year-old *B. platyphylla* Suk. Suspension cultures were established and cultivated on optimized Nagata-Takebe (NT) medium supplemented with 0.1 mgL^{-1} 6-benzyladenine (6-BA), 0.01 mgL^{-1} thidiazuron (TDZ), and 20 gL^{-1} sucrose, at an interval of 7 ~ 10 days. The medium pH was adjusted to 5.6 with 1 M NaOH before autoclaving. Although not shown in this report, previous work demonstrated a considerable variation in the amount of triterpenoids with subcultures. For this reason, experiments were done in stabilized cultures, that is to say when triterpenoid production was similar after four consecutive subcultures.

A single stock culture grown in a 1000 ml Erlenmeyer flask was used as inoculum for the experimental flasks. All experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of the corresponding liquid media with 20 gl^{-1} sucrose and inoculated with 4.0 g fresh weight of 8-day-old cell suspension cultures. The Erlenmeyer flasks were incubated on a rotary shake (110 rpm) at 25°C. Illumination was regulated so as to give 14 h of light (photophase 06.00 - 20.00 h) provided by fluorescent tubes (mixing Osram fluora and Osram daylight types) with a photon flux density (400 - 700 nm).

Chitosan

Stock solutions at 50 $mgml^{-1}$ of water-soluble chitosan (minimum 85% deacetylation, average molecular weight 5 KDa, Shandong, Aokang Bio-Technology Co., Ltd.) were prepared by dissolving them in distilled water and filter sterilized.

Dry cell weight and triterpenoid determination

For dry cell weight determination, cells were harvested and collected by centrifugation at 3000 rpm for 15 min and washed with distilled water. The fresh cells were dried at 60 ± 2°C to a constant dry weight. Extraction of triterpenoids was done with 95% methanol (Fan et al., 2009a). Extract was thereafter analyzed by ultraviolet spectrophotometer under 510 nm wavelength.

Enzyme extraction and activity assay

Fresh cells were frozen and homogenized in an ice bath with extraction buffer which consists of 0.05 M Na phosphate buffer (pH 7.0), 2% polyvinylpyrrolidone, 0.25 M sucrose, 2 mM EDTA, 5 mM dithiothreitol and 5 mM $MgCl_2$. The homogenate was filtrated through a 4-layer of nylon cloth and the filtrate was centrifuged at 13000 rpm for 15 min at 4°C. The supernatant was used for enzyme assays. Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) activity was based on the PAL conversion of L-phenylalanine to cinnamic acid using a modified method of Zhang (2002). Superoxide dismutase (EC 1.15.1.1) activity was assessed by monitoring the inhibition of photochemical reduction by nitro blue tetrazolium reaction (NBT), according to the method of Beyer and Fridovich (1987). Endochitinases (EC 3.2.1.14) and exochitinases (EC 3.2.1.52) were determined following the method of Jeuniaux (1966), using colloidal chitin as substrate. Colloidal chitin was prepared by the method as described by Sandhya (2004).

Statistical analysis

All experiments were repeated three times. The data obtained were statistically analysed by SPSS (8.0) Means and standard error were calculated from three replicates.

RESULTS

Elicitor dose

In preliminary experiments, chitosan elicitor was screened at various doses (0 ~ 500 mgL^{-1}) to optimize the concentrations to obtain maximum triterpenoid accumulation (Figure 1). Addition of chitosan elicitor to *B. platyphylla* Suk. cells enhanced cell dry weight and triterpenoid production with an increase in the dose up to 100 mgL^{-1}. Above this level, decreased cell growth and triterpenoid accumulation were observed. The maximum triterpenoid production of the treated cell cultures was 202.75 mgL^{-1} under 100 mgL^{-1} elicitor dose, which is 2.6-fold higher than that of the control without elicitation (78.72 mgL^{-1}). On the basis of the results the concentration of 100 mgL^{-1} in the medium was chosen for further experiments.

Culture age

The response to elicitation is dependent on growth phase of the culture, which not only affects the quantitative response but also the product pattern in general (Komaraiah et al., 2002).Therefore, *B. platyphylla* Suk. cell cultures at various ages (3, 8 and 13-day-old) were

treated by the selected chitosan elicitor of 100 mgL^{-1}, and then dry weight and triterpenoid accumulation were analyzed after a five-day induction (Figure 2). It was observed that the maximum dry weight (9.90 gL^{-1}) and triterpenoid production (230.01 mgL^{-1}) were obtained by eliciting 13-day-old cells. However, the triterpenoid weight under chitosan treatment at 8 days (31.57), 13 days (24.24) and 3 days (18.49) were different. So, the optimum age of the culture for elicition was on the 8th day.

Incubation time

The effect of treatment time with elicitors is presented in the Figure 3. Effective induction of triterpenoid accumulation in culture cells varied from 1 to 7 days. A maximum triterpenoid yield of 239.77 mgL^{-1} DW was obtained after 3 days, whereas triterpenoid production declined during an extended time of incubation. This reveals that the duration of cultivation in the elicitor was rather important with respect to triterpenoid production.

Effect of chitosan on defense enzyme elicitation

Activation of defense responses of *B. platyphylla* Suk. cells to chitosan elicitation was assessed by determining phenylalanine ammonia-lyase (PAL), superoxide dismutase (SOD), and chitinase activities at different time intervals from 0.5 to 72 h after treatment (Figure 4). PAL activity slowly increased with the extension of elicited time in *B. platyphylla* Suk. cells, and the maximum was reached 24 h after elicitation, at which time it was 2.0-fold higher than in non-elicited cells. SOD activity was transiently increased in elicited cells at 4 h, being 2.1-fold higher than control. Chitinase activities was also transiently increased after treatment, with a peak at 6 h, though the enhancement of endochitinase (EDC) was different from that of exochitinase (EOC), being 1.6 and 3.0 fold higher than control, respectively.

DISCUSSION

Our results demonstrate that chitosan is effective in enhancing triterpenoid biosynthesis in *B. platyphylla* Suk. suspension cell cultures. Triterpenoid production of treated cell cultures reached 202.75 mgL^{-1}, which is 2.6-fold higher than that of control (78.72 mgL^{-1}). The results also show that elicitor dose, cell culture age and incubation time significantly influence biomass growth and triterpenoid production in *B. platyphylla* Suk. cells. This is in agreement with that reported for plumbagin production in suspension cultures of *Plumbago rosea* L. and phenylethanoid glycosides biosynthesis in *Cistanche deserticola* cell suspension cultures elicited with chitosan (Komaraiah et al., 2002; Cheng et al., 2006), confirming

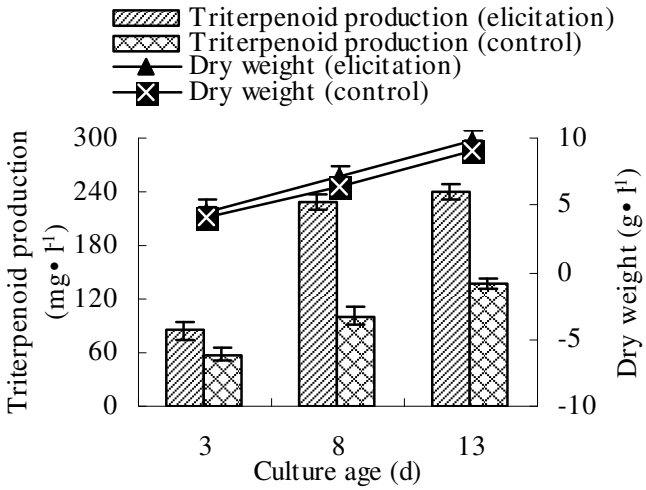

Figure 2. Effect of cell culture age on cell growth and triterpenoid production. Chitosan was added at the optimum dose level of 100 mgl^{-1}, and the cells harvested after five days of incubation. Control cells received the same amount of distilled water. Data shown are mean of three replicates and ±S.D. values presented as error bars.

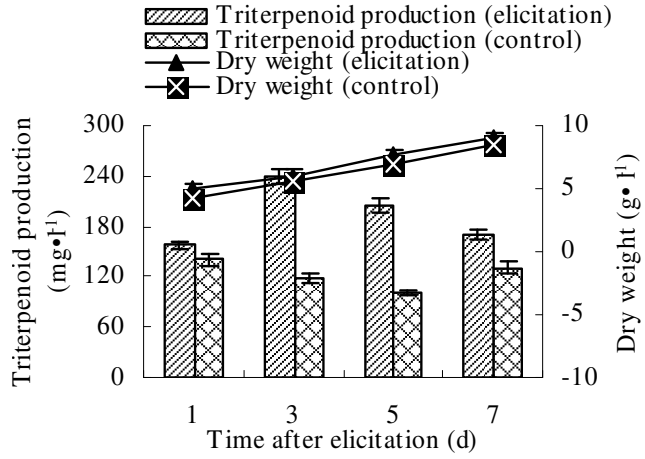

Figure 3. Effect of incubation time on cell growth and triterpenoid production. Chitosan was added at the optimum dose level of 100 mgl^{-1} and at the optimum age of the culture cells (8 days). Control cells received the same amount of distilled water. All cells were harvested after five days of incubation. Data shown are mean of three replicates and ±S.D. values, presented as error bars.

that optimization of induction conditions is rather important with respect to secondary metabolite production.

Defense reactions that result from elicitors are usually composed of a multitude of biochemical events including Oxidative burst, accumulation of wall-bound phenolic compounds, induction of enzymes for lignin and phytoalexin synthesis, synthesis of hydrolytic enzymes like chitinase and β-1,3-glucanase, and accumulation of secondary metabolite (Qi et al., 2008). Among elicited enzyme activities, phenylalanine ammonia-lyase (PAL) is

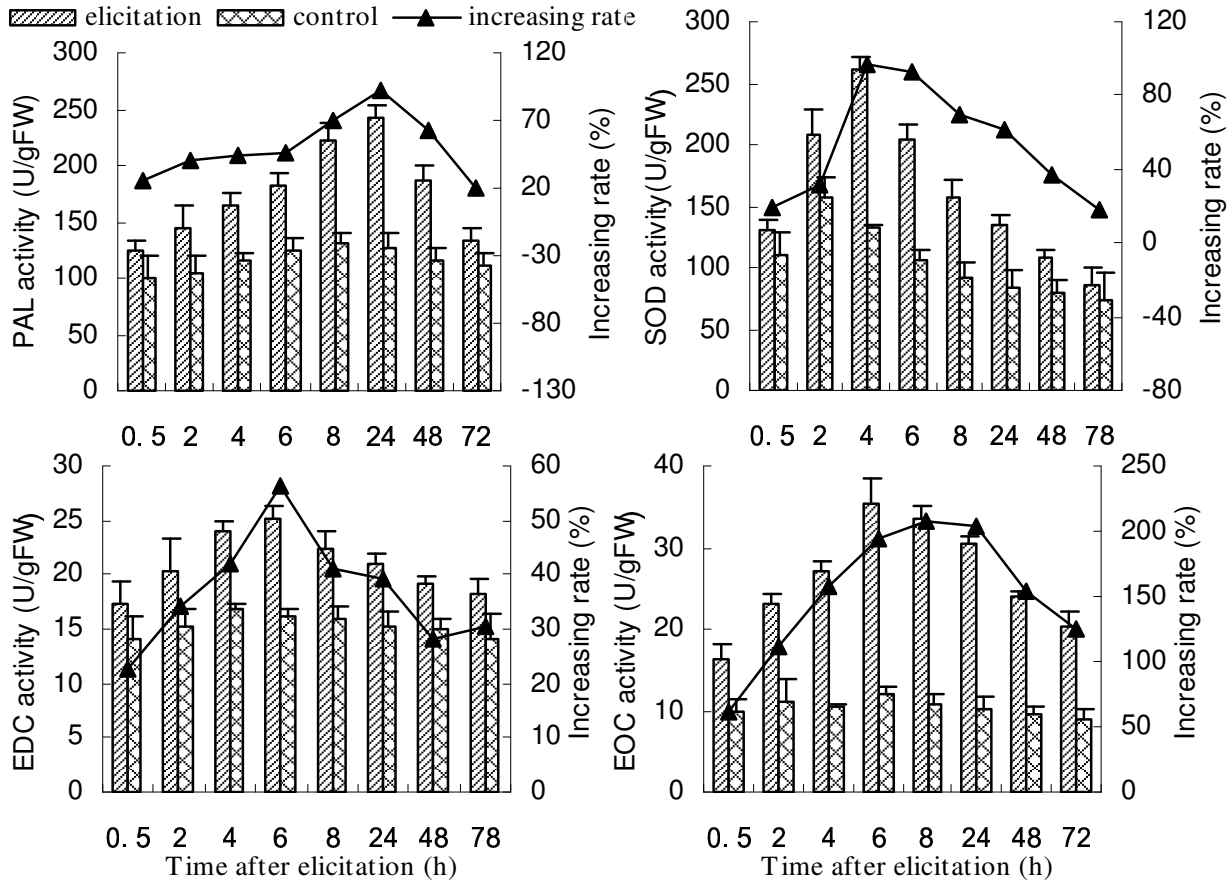

Figure 4. Time course of specific activities of phenylalanine ammonia-lyase (PAL), superoxide dismutase (SOD), endochitinase (EDC) and exochitinase (EOC) from cell suspension cultures of *Betula platyphylla* Suk. after addition of 100 mg l^{-1} of chitosan. The cells were harvested at 0.5, 2, 4, 6, 8, 24, 48 and 78 h, respectively. Values are means of triplicate results and error bars represent standard errors.

the first to be activated, catalyzing the initial step of the phenylpropanoid pathway, that regulates the production of precursors for lignin biosynthesis and other phenolic defensive compounds in plant cells (Liu and Cheng, 2008). Superoxide dismutase (SOD) is instead activated to scavenge the overproduction of reactive oxygen species (ROS) produced during the initial oxidative burst following elicitation (Iriti and Faoro, 2007). Thus, the increased activities of SOD and PAL we found suggest that a typical defense reaction has been activated by chitosan elicitation (Chakrabortya et al., 2009), and this is possibly responsible for enhanced triterpenoid biosynthesis.

Plant chitinases, which are induced by pathogen infection and elicitor treatments, are pathogenesis-related (PR) proteins that play a role in the defense system in addition of being useful markers for host defense responses (Shinya et al., 2007). Besides defense responses, plant chitinases are involved in tolerance, to abiotic stresses, symbiosis, and plant development (Wiweger et al., 2003). Because individual plant species have many different chitinases (Brunner et al., 1998; Truong et al., 2003), it is important to understand the regulation and functions of different chitinase genes. In this work, we found that chitosan differentially affected chitinases activity, with endochitinase (EDC) and exochitinase (EOC) raised 1.6 and 3.0 fold, respectively, in comparison with non-elicited control. The significance of this differential raising is not known and needs further investigation. Nevertheless, the fact that chitosan raised chitinases activity in *B. platyphylla* Suk. cell suspension cultures confirms its capability in eliciting defense mechanisms also in this plant species. In turn, this suggests that the observed enhanced triterpenoid biosynthesis may be part of these mechanisms.

In conclusion, the possibility of enhancing significantly the biosynthesis of important triterpenoids such as those of *B. platyphylla* Suk. in cell cultures by elicitation with chitosan, a cheap natural polysaccharide, deserves particular attention in view of a possible large scale practical application. In this regard, studies are underway to demonstrate the possibility of further increase in the production of triterpenoids with repeated elicitation cycles, in both shake flasks and bioreactors.

ACKNOWLEDGEMENT

Supported by China Postdoctoral Science Foundation (No. 20080440136), the Fundamental Research Funds for the Central Universities (No. DL09BA05) and Natural Science Foundation of Heilongjiang Province (No. ZD200918).

REFERENCES

Alakurtti S, Taru MK, Salme K (2006). Pharmacological properties of the ubiquitous natural product betulin European. J. Pharmaceut. Sci. 29: 1-13

Beyer WF, Fridovich I (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in condition. Anal. Biochem. 161: 559-566

Brunner F, Stintzi A, Fritig B (1998). Substrate specificities of tobacco chitinases. Plant J. 14: 225-234

Chakrabortya M, Karunb A, Mitraa A (2009) Accumulation of phenylpropanoid derivatives in chitosan-induced cell suspension culture of *Cocos nucifera*. J. Plant Physiol. 166: 63-71.

Chang JH, Shin JH, Chung IS (1998). Improved menthol production from chitosan-elicitedsuspension culture of *Mentha piperita*. Biotechnol. Lett. 20(12): 1097-1099.

Cheng XY, Zhou HY, Cui X (2006). Improvement of phenylethanoid glycosides biosynthesis in *Cistanche deserticola* cell suspension cultures by chitosan elicitor. J Biotechnol 121: 253-260

Eilert U, Ehmke A, Wolters B (1984) Elicitor-induced accumulation of acridone alkaloid epoxides in *Ruta graveolens* suspension cultures. Planta Med. 50: 508-512.

Fan GZ, Zhan YG, Wang B (2009b) Effects of light on growth and triterpenes content in callus of *Betula platyphylla*. J. Northeast Fores. Univ. 1: 1-3

Fan GZ, Zhang YG, Li K (2009a). Preliminary optimization the extration conditions of triterpene in callus of *Betula platyphylla* Suk. Chin. Agric. Sci. Bull. 25(02): 55-58

Iriti M, Faoro F (2007). Chitosan as a MAMP, searching for a PRR. Plant Signaling and Behaviour 4: 1-3.

Iriti M, Faoro F (2009). Review of innate and specific immunity in plants and animals. Mycopathologia 164: 57-64.

Jeuniaux C (1966) Methods in Enzymology. Academic press, New York 8: 644-650.

Jing Z, Li H, David L (2005). Inhibition of HIV-1 maturation via drug association with the viral gag protein in immature HIV**-**1 particles. J. Biol. Chem. 280: 42149-42155

Komaraiah P, Amrutha RN, Kishor PBK (2002). Elicitor enhanced production of plumbagin in suspension cultures of *Plumbago rosea* L. Enzyme Microb. Technol. 31: 634-639.

Liu CZ, Cheng XY (2008). Enhancement of phenylethanoid glycosides biosynthesis in cell cultures of *Cistanche deserticola* by osmotic stress. Plant Cell Rep. 27: 357-362.

Nahálková JJ, Gemeiner P, Blanárik P (1998). Elicitation of plumbagin by chitin and its release into the medium in Drosophyllum lusitanicum Link. suspension cultures. Biotechnol. Lett. 20(9): 841-845

Putalun W, Luealon W, De-Eknamkul W (2007). Improvement of artemisinin production by chitosan in hairy root cultures of *Artemisia annua* L. Biotechnol. Lett. 29: 1143-1146

Qi FH, Zhan YG, Jing TZ (2008). A review on elicitors and their regulation on secondary metabolites in Plant Cell Culture. Natl. Prod. Res. Dev. 20: 568-573.

Sánchez-Sampedro MA, Fernández-Táarrago J, Corchete P (2005). Yeast extract and methyl jasmonate-induced silymarin production in cell cultures of *Silybum marianum* (L.) Gaertn J. Biotechnol. 119: 60-69.

Sandhya C, Adapa LK, Nampoothiri KM (2004). Extracellular chitinase production by Trichoderma harzianum in submerged fermentation. J. Basic Microbiol. 44: 49-58.

Sanford PA, Hutchings GP (1987). Chitosan - a natural, cationic biopolymer: commercial applications. In: Yapalma M (Ed) Industrial Polysaccharides: Genetic Engineering, Structure/Property Relations and Applications, Elsevier.

Shinya T, Hanai K, Gális I (2007). Characterization of NtChitIV, a class IV chitinase induced by β-1,3-, 1,6-glucan elicitor from Alternaria alternata 102: Antagonistic effect of salicylic acid and methyl jasmonate on the induction of NtChitIV. Biochem Biophys Res Commu 353: 311-317.

Truong NH, Park SM, Nishizawa Y (2003). Itoh, Structure, heterologous expression, and properties of rice (*Oryza sativa* L.) family 19 chitinases. Biosci. Biotechnol. Biochem. 67: 1063-1070

Wang B, Fan GZ, Zhan YG (2008a). Effects of different carbon sources on callus growth and accumulation of triterpenoids in Birch (*Betula platyphylla*). Plant Physiol. Comm. 44(1): 97-99.

Wang B, Fan GZ, Zhan YG (2008b). Effects of different medium types and plant growth regulator combinations on accumulation of triterpenoids in Birch (*Betula platyphylla*) Calli. Scientia Silvae Sci. 44(10): 153-158.

Wen ZM, Martin DE, Bullock P (2007). Glucuronidation of anti-HIV Drug candidate bevirimat: identification of human UDP-glucuronosyltransferases and species differences. Drug Metabol. Dispos. 35: 440-448.

Wiweger M, Farbos I, Ingouff M (2003). Expression of Chia4-Pa chitinase genes during somatic and zygotic embryo development in Norway spruce (*Picea abies*): similarities and differences between gymnosperm and angiosperm class IV chitinases. J. Exp. Bot. 54: 2691-2699.

Zhang CH, Mei XG, Liu L (2000). Enhanced paclitaxel production induced by the combination of elicitors in cell suspension cultures of *Taxus chinensis*. Biotechnol. Lett. 22: 1561-1564.

Zhang CH, Wu JY, He GY (2002). Effects of inoculum size and age on biomass growth and paclitaxel production of elicitor-treated *Taxus yunnanensis* cell cultures. Appl. Microbiol. Biotechnol. 60: 396-402.