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Review

Bacteriocins of Gram-positive bacteria: Features and biotherapeutic approach

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Bacteriocins are potent antimicrobial peptides produced by every bacterial and archeal species reported to date. The most studied are bacteriocins produced by lactic acid bacteria (LAB) and many species of *Bacillus*. Knowledge on the classification, biosynthesis and transport of these peptides is changing continually because the discovery and characterization of new bacteriocins increases, thus, the research reports increase at the same rate. The bacteriocins are considered the most promising molecules with enormous possibilities and realities for the design of improved antibiotics possessing specific characteristics, mostly against antibiotic resistant bacteria. Here, current information on the generalities, classification proposals, biosynthesis and transport systems involved in the bacteriocins secretion is review. Finally, this review will focus on the new approaches for its application in veterinary medicine and human health.

Key words: Bacteriocin, biotherapeutic, resistant bacteria, human health.

INTRODUCTION

Bacteriocins are antimicrobial protein produced by all major lineages of bacteria and archeal species studied to date. Technically are defined as antimicrobial peptides ribosomally synthesized, which may or may not be post-translationally modified and its spectrum of action. Bacteriocins produced by lactic acid bacteria (LAB) are the most studied, however, there are also species of *Bacillus*, Gram-positive aerobic organism that can resist environmental stress by forming endospores (Kumar et al., 2011), and also, is capable of producing significant quantities of powerful bacteriocins (Abriouel et al., 2011).

Characteristics of bacteriocins from Gram-positive bacteria are very well known, such as the synthesis regulated by particular systems for each bacteriocins class. The synthesis is not lethal to the producer cell due to the transport generally is mediated by inner transport systems like the Sec System and the ABC-transporter System (Gutiérrez et al., 2006). Also has been reported

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extensively its spectrum of antimicrobial action, including both narrow spectrum with limited activity and primarily only useful against related species of microorganisms, and broad spectrum active against both Gram-positive and Gram-negative organisms and some fungi (Salazar-Marroquín et al., 2016).

CLASSIFICATION APPROACH OF BACTERIOCINS

In recent years, there has been booming research on bacteriocins, and their classification by size, spectrum of activity, mode of action, chemical composition, stability, biosynthesis and other factors. Actually, the more inclusive classification of these widely heterogeneous peptides considers all the above points, subdivided into classes including the subclasses previously reported for unmodified bacteriocins synthesized by LAB (Cotter et al., 2013).

Class I: Lantibiotics. Small peptides less than 5 kDa, active at membrane level and containing some unusual amino acids such as lanthionine, β-methyl-lanthionine and deshirdro alanine formed due to subsequent modifications to the process of translation. The formation of uncommon amino acids is explained by dehydration of the amino acids serine and threonine, with the subsequent addition of sulfur of cysteine and the formation of dehydroamino double bonds (Chen and Hoover, 2003). Class I is subdivided according to its antimicrobial activity and chemical structure into two types A and B (Cotter et al., 2005).

Class I A: Positively charged elongated peptides that act at the level of the bacterial membrane forming pores, promoting the dissipation of the membrane potential and lacking in small metabolites of sensitive cells.

Class I B: Negatively charged small globular peptides, the antimicrobial activity of these is linked to the inhibition of specific enzymes.

Class II: No lantibiotics. Peptides less than 10 kDa in molecular weight, thermostable, do not undergo posttranslational modifications. Three classes are released from this group, shown below.

Class IIa: Active peptides against *Listeria* sp. contain a consensus sequence in the N-terminal region - TGNGVXC.

Class II b: Complex of two peptides necessary for better antimicrobial activity consisting in pore formation.

Class II c: Small peptides, thermoset, unmodified, which are transported by a leader peptide.

Class II d: Novel leaderless bacteriocins, atypical in the sense that they are synthesized without an N-terminal leader sequence and with unique biosynthetic mechanisms (Oman and van der Donk, 2010).

Class III: Bacteriolicinas, with a molecular weight greater than 30 kDa, are thermolabile and its action consist of the hydrolysis of the cell wall of sensitive bacteria.

Clas IV: Circular bacteriocins. Peptides covalently linked head to tail and share a common structural motif (Martin-Visscher et al., 2009). The common motif has four or five conserved α helices enclosing a compact hydrophobic core. The most studied circular bacteriocin is enterocin AS-48 from *Enterococcus faecalis*. Others circular bacteriocins are butyrivbriocin AR10, gassericin A, circularin A, subtilosin A, uberloysin, reutericin and lactocyclicin Q (Nes et al., 2007)

BACTERIOCIN BIOSYNTHESIS

Commonly bacteriocins are synthesized in a particular way as an inactive peptide with an N-terminal leader attached to a C-terminal called pre-peptide or rather that pre-bacteriocin. The pathway includes the pre-bacteriocin production, some reactions of particular modification and late the cleavage of the leader peptide and then, the translocation of the pro-bacteriocin across the cell membrane.

Bacteriocins genetic determinants encoding for the synthesis of bacteriocins are grouped in one or two operons consisting of different components, located on plasmids, in the chromosome, or in transposons inserted in the chromosome (Drider et al., 2006; Wirawan et al., 2007). The components includes:

1) The structural gene, encodes the pre-probacteriocin, containing an N-terminal, the leader sequence double-glycine type or peptide signal type sequences type. Double-glycine-type leader is characterized by two conserved glycines at its C-terminus, recognized by ABC transporters for processing the leader sequence and secretion of the mature bacteriocin to the extracellular medium.

The signal peptide type sequences (SP) enable the processing and secretion of bacteriocins through the general transport path (GSP) (Driessen and Nouwen, 2008).

2) Immunity gene, encodes small proteins, with sizes of approximately 51 to 154 amino acids, which protects the producing strain of the bacteriocin itself.

3) Genes encoding proteins responsible for processing, transport and secretion of the pre-probacteriocin.

4) Genes encoding the enzymes responsible for post-translational modifications of the probacteriocin.

5) Genes encoding components involved in the regulation of the synthesis. The production of bacteriocins is considered an adaptive response and therefore, is

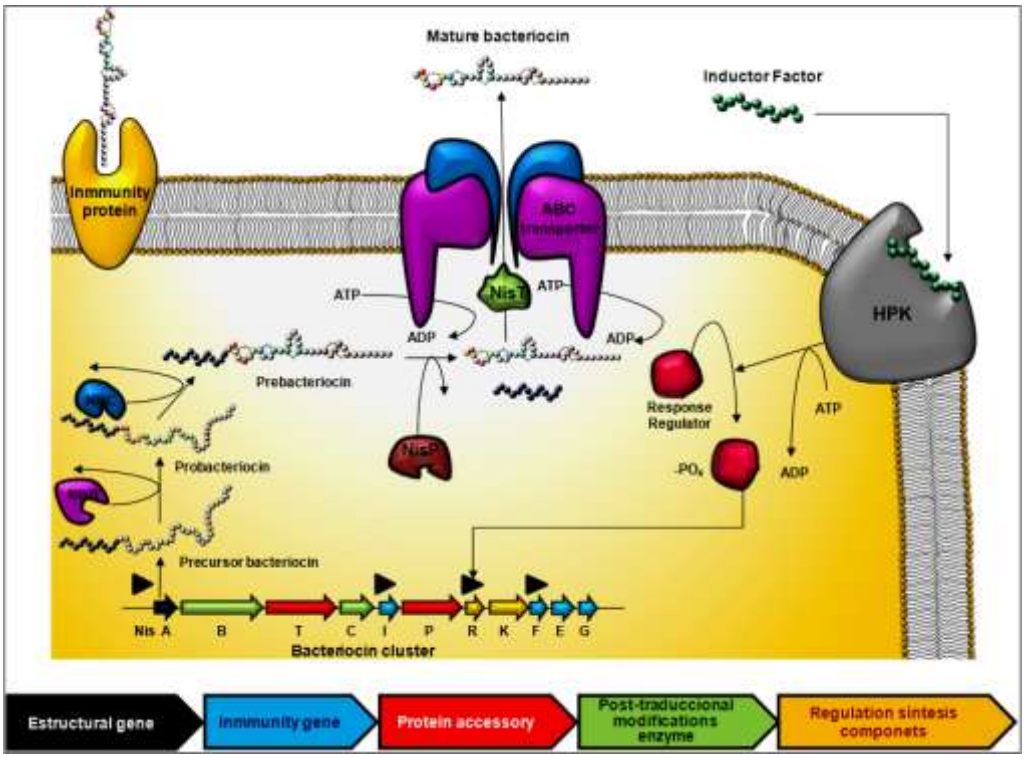

Figure 1. Genetic determinants encoding for the synthesis of bacteriocins. The structural gene encodes the pre-probacteriocin, the immunity gene encodes small proteins, genes encoding proteins for processing, transport and secretion of the pre-probacteriocin, genes encoding the enzymes for post-translational modifications and genes encoding components involved in the regulation of the synthesis.

regulated in function of certain environmental factors (Skaugen et al., 2003). The regulation process undergo by a signal transduction systems of three components that includes the inductor peptide (IP), an sensor histidine protein kinase (HPK) and response regulatory protein (RR). The complete process for bacteriocin production and secretion is shows in Figure 1. Two models have been proposed to explain the induction process, the inducing peptide and signal transduction mechanism.

The inducer peptides (IPs) are small cationic molecules that form an amphiphilic α helix and are the signal of the regulatory systems or "quorum sensing" that control the biosynthesis of certain bacteriocins. According to the model, the IP is produced constitutively in small amounts, thereby is accumulated progressively during cell growth, and when the levels required to exert the induction there is an increase in the expression of genes of the bacteriocin gene cluster.

The second model to explain the induction proposes that IP occurs at a level below that required for the self-induction, and in diverse environmental factors temporarily increases its production, so when required levels are exceeded, again, it induces its own synthesis and the remaining genes from the bacteriocin gene cluster (Figure 2) (Straume et al., 2007).

TRANSPORT SYSTEMS

Most proteins are synthesized as inactive precursors or pre-proteins with a signal sequence essential for the cell to recognize and transport an extracellular protein to the outside by two systems, the ABC-Transporter System or Dedicated Transportation System (DTS) and the General Secretory Pathway (GSP) or Sec System.

ABC-transport system or dedicated transportation system

ATP-binding cassette (ABC) proteins make up one of the largest superfamilies of proteins and are found in all living organisms. Most are membrane transporters that couple ATP hydrolysis to import or export of a large variety of substrates (Orelle et al., 2008).

A classic ABC-transporter includes four domains, two transmembrane domains (TMD) and two ATP-binding domains. Transmembrane domains are N-terminal, hydrophobic, and are integrated into the membrane, while the ATP-binding domains are C-terminal, hydrophilic, and are associated with the cytoplasmic face of the cell membrane. Typically, peptides function as four

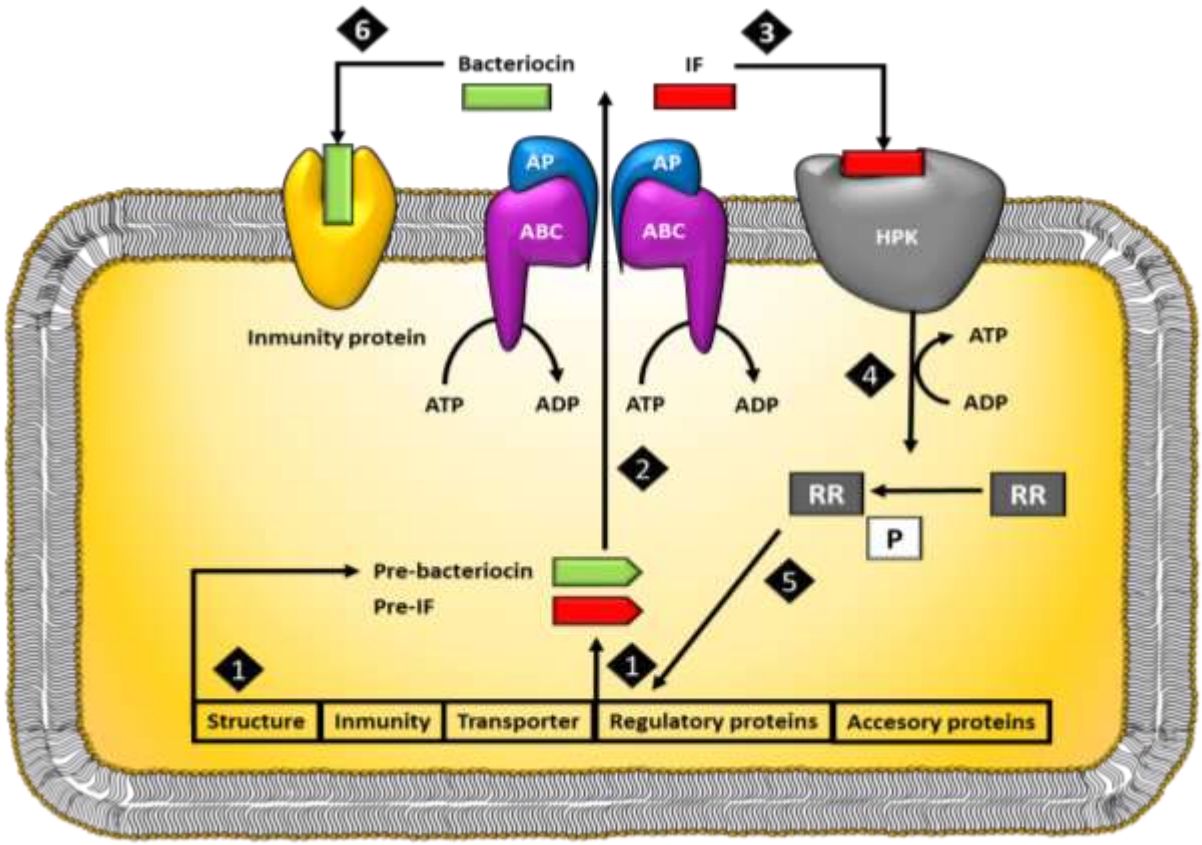

Figure 2. Biosynthesis of class II bacteriocins (modified of Chen and Hoover, 2003).

independent domains; however, in some cases, may act as a polypeptide complex (Locher, 2009).

The ABC-transporter system is also known as dedicated transport system (DTS), usually specific for a protein or group of proteins of the same family. The carrier protein (ABC) (1), the hydrophilic C-terminal with binding sites for ATP (2) and the accessory protein (PA) interacting with ABC type transporter are shown in Figure 3 (Kodali et al., 2013).

General secretory pathway (Sec)

Protein complexes of the Sec family are found universally in prokaryotes and eukaryotes. The peptides and proteins synthesized with an N-terminal signal peptide type (SP) are processed and secreted by the general secretory pathway (GSP). Secretion of peptides requires the participation of several components, the majority of the characteristics reference the translocase of *E. coli*. However, homologous systems have been described in *Bacillus subtilis* (Kuipers et al., 2006). The translocase in *E. coli* consists of three integral inner membrane proteins, SecYEG, and the cytoplasmic ATPase, SecA. SecA recruits SecYEG complexes to form the active translocation channel, and proteins are translocated through the SecY channel. A long α-helix in SecA is important for coupling of ATPase activity to protein translocation (Sanganna Gari et al., 2013).

PROMISING ACTION OF BACTERIOCINS TO BE CONSIDERED AS BIOTHERAPEUTICS

Bacteriocins are distinguished from antibiotics by two main characteristics: bacteriocins are ribosomally synthesized and have a relatively narrow killing spectrum (Riley and Wertz, 2002), and may be valuable biotherapeutic tools.

The biotherapeutic are medicines derived from proteins and/or different molecules produced by eukaryotic cells, bacteria and viruses, which have been applied in comprehensive and innovative treatments worldwide (Dobson et al., 2012). Some treatments use class I (nisin) and unmodified class IIa bacteriocins like pediocin, enterocin and divergicin for biotherapeutic application.

Pediocin PA-1 confirm its effect against *Listeria monocytogenes*, pathogenic bacterium that causes listeriosis, a serious infection spread by eating contaminated foods. The provision of purified pediocin

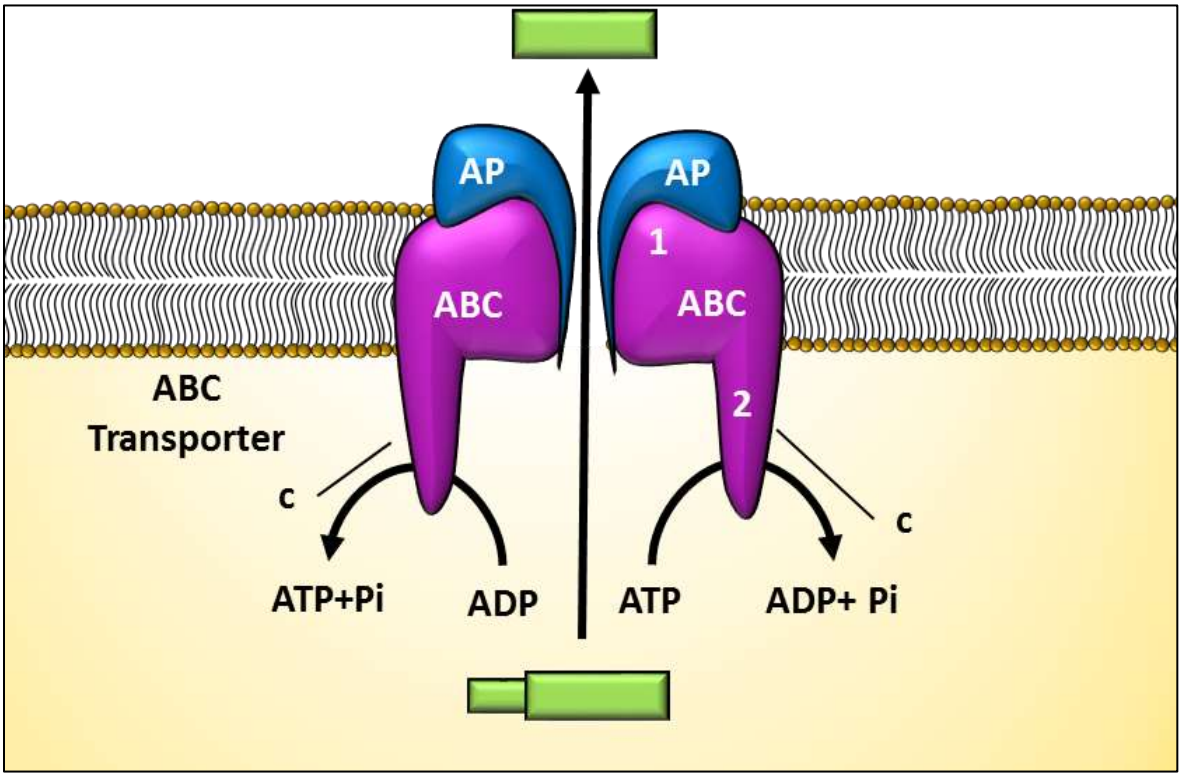

Figure 3. ABC-Transport System in Gram-positive bacteria (Adapted from Kodali et al., 2013).

intraperitoneally in mice as model systems infected with *L. monocytogenes*, reduces 100 times the total count of intestinal *Listeria*. This research confirms the effectiveness of treatment for the prevention of listeriosis by the action of the bacteriocin (Dabour et al., 2009). The enterocin E50-52 synthesized by *Enterococcus faecium* (NRRL B-30746) has been proven effective *in vitro* against *Staphylococcus aureus* resistant to methicillin (MRSA), showing capability for application as an alternative treatment without generating resistance antibiotics (Dobson et al., 2012).

The same bacteriocin in minimum inhibitory concentration (MIC) from 0.025 to 32 mg/mL was tested against *Campylobacter jejuni, Yersinia* spp., *Salmonella* spp., *E. coli* O157: H7, *Shigella dysenteriae, Morganella morganii, Staphylococcus* spp. and *Listeria* spp, showing significant results, for example, orally administered reduce up to 100, 000 fold for *C. jejuni* and *Salmonella enteritidis* in the intestine, and also minimizes the survival of *S. enteritidis* in the liver (Svetoch et al., 2008a, b).

Use of enterocin S760 for prophylaxis in dose 50 mg/kg during 10 days prevented lethal infection in 100% of mice, whereas its use for treatment cured 70% of animals with salmonellosis (Svetoch et al., 2010).

L. monocytogenes is a bacterial pathogen responsible for listeriosis, a foodborne disease characterized by septicemia and abortion in pregnant women, and it is also responsible for gastroenteritis in healthy individuals and for a severe invasive disease in immunocompromised patients. *In vivo,* the activity of divergicin V41 against *L. monocytogenes* EGDe administered intravenously in mice confirms its antilisterial activity and growth experiments revealed the reduce bacterial growth (Rihakova et al., 2010). The *L. monocytogenes* EGD-e contains a luciferase-based vector, pPL2lux, and use of this vector to study gene expression in *L. monocytogenes*. pPL2lux is a derivative of the listerial integration vector pPL2 and harbors a synthetic luxABCDE operon encoding a fatty acid reductase complex (LuxCDE) involved in synthesis of the fatty aldehyde substrate for the bioluminescence reaction catalyzed by the LuxAB luciferase.

Nisin F is a new lantibiotic bacteriocin produced by a *L. lactis* subsp. *lactis* Isolate from freshwater catfish (*Clarias gariepinus*), active against *S. aureus, Staphylococcus carnosus, L. curvatus, L. plantarum* and *L. reuteri.* The effectiveness of nisin F has been experimentally tested *in vivo* in rats that were infected with *S. aureus* (De Kwaadsteniet et al., 2008). Rats treated with nisin F administered intranasally (80-320 AU/mL) remained healthy. This preliminary evidence in animals should be confirmed in humans to control respiratory tract infections caused by *S. aureus* (De Kwaadsteniet et al., 2009).

The ability of nisin F to control *S. aureus* infection in the peritoneal cavity was studied in mice, and the suppresion

of growth of *S. aureus* was confirmed in the peritoneal cavity for at least 15 min (Brand et al., 2010). The use of bacteriocins such as biotherapeutics has been demonstrated not only in bacteriocins synthesized by lactic acid bacteria, besides there are reports of successful therapy with *Bacillus* bacteriocins. *Bacillus thuringiensis* is one of the most profitable entomopathogenic bacteria that has been used as a biopesticide due to its ability to synthesize insecticidal crystal proteins during sporulation and vegetative insecticidal proteins during the vegetative phase of growth. Also, it is important in this review because it may expand its potential by producing bacteriocins, thuricins named according to the subspecies that can synthesize these antimicrobial proteins (Chaabouni et al., 2012; De la Fuente-Salcido et al., 2013; Huang et al., 2014).

The most successful example is reported for thuricin CD, the bacteriocin synthesized by *B. thuringiensis* strain DPC 6431 whose activity has been proven effective as a biotherapy in the treatment of diarrhea associated with *Clostridium difficile* without collateral impact on the intestinal flora of patients treated (Rea et al., 2010, 2011). Another important field for biomedical application of bacteriocins from *B. thuringiensis* (morricin, kurstacin, kenyacin, entomocin and tolworthcin) is animal health. These bacteriocins were included in the study to determine their antagonism against pathogenic strains of *S. aureus* isolated of milk from diseased cows mastitis, confirming an high antibacterial activity (Barboza-Corona et al., 2009). Also these bacteriocins are effective against pathogens isolated from bovine clinical and subclinical mastitis, because it inhibit the growth of multiantibiotic resistance bacteria such as *Staphylococcus agnetis, Staphylococcus equorum, Streptococcus uberis, Brevibacterium stationis* and *Brachybacterium conglomeratum* (León-Galván et al., 2015).

The last examples strengthens the feasibility of the application of bacteriocins with high capacity to generate new models for future alternative treatments to antibiotics for preventing and/or reducing incidence of diseases in both human and animal infections.

Conclusion

The rise in antibiotic-resistant pathogens has increased efforts to develop new antibiotics active against the resistant bacteria. The diversity of bacteriocins is a trait that gives them numerous possibilities for application in medicine, therefore, knowledge on its structure, the steps involved in biosynthesis and transport, can lead to better understanding of the mode of action and improvement of their activity. It can also lead to the generation of new broad antimicrobial spectra peptides and increase the application of bacteriocin in human health and veterinary medicine with promising commercial potential.

Undoubtedly, the antimicrobial peptides such as bacteriocins have enough features to be recognized as the next generation of biotherapeutics to treat infections caused by multidrug resistant bacteria to traditional antibiotics.

Conflict of interest

The authors did not declare any conflict of interest.

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REFERENCES

Abriouel H, Franz CM, Omar NB, Gálvez A (2011). Diversity and applications of *Bacillus* bacteriocins. FEMS Microbiol. Rev. 35:201-232.

Barboza-Corona JE, De la Fuente-Salcido N, Alva-Murillo N, Ochoa-Zarzosa A, López-Meza JE (2009). Activity of bacteriocins synthesized by *Bacillus thuringiensis* against *Staphylococcus aureus* isolates associated to bovinemastitis. Vet. Microbiol. 138: 179-83.

Brand AM, De Kwaadsteniet M, Dicks LMT (2010). The ability of nisin F to control *Staphylococcus aureus* infection in the peritoneal cavity, as studied in mice. Lett. Appl. Microbiol. 5(6):645-649.

Chaabouni I, Guesmi A, Cherif A (2012). Secondary Metabolites of *Bacillus:* Potentials in Biotechnology. In: Sansinenea E., editor. *Bacillus thuringiensis* biotechnology. Springer; Netherlands. pp. 347-366.

Cotter PD, Ross RP, Hill C (2013). Bacteriocins-a viable alternative to antibiotics? Nature Rev. Microbiol. 11(2):95-105.

Cotter PD, Hill C, Ross RP (2005). Bacteriocins: developing innate immunity for food. Nature Rev. Microbiol. 3(10):777-788.

Chen H, Hoover DG (2003). Bacteriocins and their Food Applications. Compr. Rev. Food Sci. Food Saf. 2(3):82-100.

Dabour N, Zihler A, Kheadr E, Lacroix C, Fliss I (2009). *In vivo* study on the effectiveness of pediocin PA-1 and *Pediococcus acidilactici* UL5 at inhibiting *Listeria monocytogenes*. Int. J. Food Microbiol. 133(3):225-233.

De Kwaadsteniet M, Doeschate K, Dicks LMT (2009). Nisin F in the treatment of respiratory tract infections caused by *Staphylococcus aureus*. Lett. Appl. Microbiol. 48:65-70.

De Kwaadsteniet M, ten Doeschate K, Dicks LMT (2008). Characterization of the structural gene encoding Nisin F, a new lantibiotic produced by a *Lactococcus lactis* subsp. *lactis* isolate from freshwater catfish (*Clarias gariepinus*). Appl. Environ. Microbiol. 74(2):547-549.

De la Fuente-Salcido NM, Casados-Vázquez LE, Barboza-Corona JE (2013). Bacteriocins of *Bacillus thuringiensis* can expand the potential of this bacterium to other areas rather than limit its use only as microbial insecticide Can. J. Microbiol. 59:515-522.

Drider D, Fimland G, Héchard Y, McMullen LM, Prévost H (2006). The continuing story of class IIa bacteriocins. Microbiol. Mol. Biol. Rev. 70(2):564-82.

Driessen AJM, Nouwen N (2008). Protein translocation across the bacterial cytoplasmic membrane. Annu. Rev. Biochem. 77:1-25.

Dobson A, Cotter PD, Ross RP, Hill C (2012). Bacteriocin Production: a Probiotic Trait? Appl. Environ. Microbiol. 78(1):1-6.

Gutiérrez J, Larsen R, Cintas LM, Kok J, Hernández PE (2006). High-level heterologous production and functional expression of the sec-

dependent enterocin P from *Enterococcus faecium* P13 in *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 17:1-11.
Huang T, Zhang X, Xiaoyu Su JP, Jin X, Guan X (2014). Aerobic Cr (VI) reduction by an indigenous soil isolate *Bacillus thuringiensis* BRC-ZYR2. Pedosph. 24:652-661.
Kuipers A, Wirenga J, Rink R, Kluskens LD, Driessen AJM, Kuipers OP, Moll GN (2006). Sec-mediated transport of posttranslationally dehydrated peptides in *Lactococcus lactis.* Appl. Environ. Microbiol. 72:7626-7633.
Kumar A, Prakash A, Johri BN (2011). *Bacillus* as PGPR in Crop Ecosystem. In: MAHESHWARI, D. K. (ed.) Bacteria in Agrobiology: Crop Ecosystems. Springer Berlin Heidelberg.
León-Galván MF, Barboza-Corona JE, Lechuga-Arana A, Valencia-Posadas M, Aguayo D, Cedillo-Pelaez C, Martínez-Ortega EA, Gutierrez-Chavez A (2015). Molecular detection and sensitivity to antibiotics and bacteriocins of pathogens isolated from bovine mastitis in family dairy herds of central Mexico. BioMed Res. Internat. ID 615153, 9 pages .
Locher KP (2009). Review:Structure and mechanism of ATP-binding cassette transporters. Philosophical Transactions of the Royal Society of London - Series B: Biol. Sci. 364(1514):239-245.
Martin-Visscher LA, Gong X, Duszyk M, Vederas JC (2009). The three-dimensional structure of carnocyclin A Reveals that many circular bacteriocins share a common structural motif. J. Biol. Chem. 284(42):28674-28681.
Kodali VP, Lingala VK, Karlapudi AP, Indira M, Venkateswarulu TC, Babu DJ (2013). Biosynthesis and potential applications of bacteriocins. J. Pure Appl. Microbiol. 7(4):2933-2945.
Nes IF, Yoon SS, Diep DB (2007). Ribosomally synthesized antimicrobial peptides (bacteriocins) in lactic acid bacteria: a review. Food Sci. Biotechnol. 16:675-690.
Oman J, van der Donk WA (2010). Follow the leader: the use of leader peptides to guide natural product biosynthesis. Nature Chem. Biol. 6:9-18.
Orelle C, Ayvaz TM, Everly R, Klug CS, Davidson AL (2008). Both maltose-binding protein and ATP are required or nucleotide-binding domain closure in the intact maltose ABC transporter. PNAS.105(35):12837-12842.
Rea MC, Dobson A, O'Sullivan O, Crispie F, Fouhy F, Cotter PD, Shanahan F, Kiely B, Hill C, Ross RP (2011). Effect of broad-and narrow-spectrum antimicrobials on *Clostridium difficile* and microbial diversity in a model of the distal colon. Proceed. Natl. Acad. Sci. 108(1):4639-4644.
Rea MC, Sit CS, Clayton E, O'Connor PM, Whittal RM, Zheng J, Hill C (2010). Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against *Clostridium difficile*. Proceed. Natl. Acad. Sci. 107(20):9352-9357.
Rihakova J, Cappelier JM, Hue I, Demnerova K, Fédérighi M, Prévost H, Drider D (2010). *In vivo* activities of recombinant Divercin V41 and its structural variants against *Listeria monocytogenes*. Antimicrob. Agents Chemother. 54(1):563-564.
Riley MA, Wertz JE (2002). Bacteriocins: evolution, ecology and application. Annu. Rev. Microbiol. 56:117-137.
Salazar-Marroquín EL, Galán-Wong LJ, Moreno-Medina VR, Reyes-López MÁ, Pereyra-Alférez B (2016). Bacteriocins synthesized by *Bacillus thuringiensis*: generalities and potential applications. Rev. Med. Microbiol. 27(3):95-101.
Sanganna Gari RR, Frey NC, Mao C, Randall L, King GM (2013). Dynamic structure of the translocon SecYEG in membrane: direct single molecule observations. J. of Biol. Chem. 288:16848-16854.
Skaugen M, Cintas LM, Nes IF (2003). Genetics of bacteriocin production in lactic acid bacteria. In: Wood BJB, Warner PJ (eds.).Genetics of lactic acid bacteria, Kluwer Academic/Plenum Publishers, Nueva York, EE.UU.
Svetoch EA, Borzilov A, Eruslanov BV, Korobova OV, Teĭmurazov MG, Kombarova TI, Diatlov IA (2010). Use of enterocin S760 for prevention and treatment of experimental *Salmonella* infection in mice. Zh. Mikrobiol. Epidemiol. Immunobiol. (5):44-48.
Svetoch EA, Eruslanov BV, Perelygin VV, Mitsevich EV, Mitsevich IP, Borzenkov VN, Levchuk VP, Svetoch OE, Kovalev YN, Stepanshin YG, Siragusa GR, Seal BS, Stern NJ (2008a). Diverse antimicrobial killing by *Enterococcus faecium* E50-52 bacteriocin. J. Agric. Food Chem. 56(6):1942-1948.
Svetoch EA, Levchuk VP, Pokhilenko VD, Eruslanov BV, Mitsevich EV, Mitsevich IP, Perelygin VV, Stepanshin YG, Stern NJ (2008b). Inactivating methicillin-resistant *Staphylococcus aureus* and other pathogens by use of bacteriocins OR-7 and E 50-52. J. Clin. Microbiol. 46(11):3863-3865.
Straume D, Kjos M, Nes IF, Diep DB (2007). Quorum-sensing based bacteriocin production is down regulated by N-terminally truncated species of gene Drider activators. Mol. Genet. Genomics 278:283-293.
Wirawan RU, Swanson KM, Kleffmann T, Jack RW, Tagg JR (2007). Uberolysin: a novel cyclic bacteriocin produced by *Streptococcus uberis*. Microbiol 153:1619-1630.

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Molecular characterization of local strains of *Bacillus thuringiensis* in the North of Algeria

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The analysis of soil samples taken from orange groves located in four cities of Northern Algeria allowed the isolation and identification of 24 strains belonging to the group *Bacillus cereus*, based on their physiological, biochemical and molecular characters investigated. They were divided as follow: 12 strains of *Bacillus thuringiensis,* 1 strain of *Bacillus pumilis*, 4 strains of *Bacillus cereus*, 5 strains of *Bacillus subtilis* and 2 *Bacillus mycoides* strains. After the molecular characterization performed with the aid of polymerase chain reaction (PCR) analysis, it was confirmed from the parasporal bodies using a scanning electron microscope that the strains of the identified collection belong to the species, *B. thuringiensis*.

Key words: *Bacillus cereus, Bacillus thuringiensis, Bacillus pumilis, Bacillus subtilis, Bacillus mycoides*, polymerase chain reaction, scanning EM.

INTRODUCTION

Phytophagous insects contribute largely to the decrease of agricultural productivity. Their management is generally based on the use of chemical pesticides which have certain benefits, but at the expense of human health and environment (Rice et al., 2007). Likely, the massive and systematic use of these products leads to the emergence of resistant genotypes (Kranthi et al., 2001; Pray et al., 2002; Shelton et al., 2002). Biological control is defined as the reduction of pest populations by natural enemies, which are naturally present in most ecosystems and can be used voluntarily in agriculture among others, as a replacement for conventional pesticides.

Bacillus thuringiensis (Bt) commercial bio-control agents are used for the management of certain insect pests. This bacterium belonging to the *Bacillus cereus* group is a Gram-positive bacillus of about 1 µm wide and 3 to 5 microns long; it is optionally aerobic/ anaerobic with ovoid spores, not deforming, and located in a central position or subterminal, at the rate of one spore per cell (Guinebretiere et al., 2008). This spore-forming bacterium

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Table 1. Distribution of Bt isolates in samples collected from different localities of western Algeria.

Number of strain	Source
25	Orchards of orange tree (Tlemcen city)
10	Orchards of orange tree (Oran city)
12	Orchards of orange tree (Ain Defla city)
13	Orchards of orange tree (Sétif city)
2	Reference strains
	•*Bt* BMG7
	•*Bt* HD22

(Bt), well known for its insecticidal properties, is associated with its ability to produce crystal inclusions in sporulation. These inclusions are proteins encoded by cry genes and have been shown to be toxic to a variety of insects and other organisms like nematodes and protozoa (Konecka et al., 2007). The primary action of cry proteins is to lyse mid-gut epithelial cells through insertion into the target membrane and form pores (Bravo et al., 2007).

Once ingested, crystals are solubilized in the alkaline environment of mid-gut lumen and activated by host proteases (Brar et al., 2007). The cry genes of Bt strains are known to be related to their toxicity (Carozzi et al., 1991; Padidam, 1992), and identification of these genes by means of polymerase chain reaction (PCR) has been used to characterize and predict insecticidal activity of the strains (Ben-Dov et al.,1997; Hansen et al.,1998). The spectrum activity of Bt toxins continually increases as a result of the ongoing isolation of new strains around the world.

The objective of this work is to isolate and characterize local strains of *B. thuringiensis*, in order to find new specific strains in our orchards since these bacilli have the power to adapt themselves to the type of soil as well as the bioclimatic conditions of orchards in which they live. The local strains could be much active as bio-pesticide strains.

MATERIALS AND METHODS

Description of strains

Sixty strains belonging to the *Bacillus* group, isolated from orchard soils of orange trees of Tlemcen, Oran, Ain Defla and Setif cities as well as two other reference strains were used in this study (Table 1).

For every sample, 1 g of soil was put in a test tube containing 10 ml of sterile distilled water. Various concentrations (10^{-1}, 10^{-2}, 10^{-4} and 10^{-3}) were prepared and stored in the ice. To eliminate the vegetative forms, the sample was heated to 80°C for 30 min. 100 µl of each concentration was spread over Luria and Bertani medium (AFNOR, 1996) at the rate of three plates by dilution, containing La L-serine. The L-serine inhibits the growth of various species of Bacillus, except *B. thuringiensis* (Ongena and Jacques, 2007).

Identification

It was started by a microscopic study after a Gram staining. Only bacteria in the form of bacilli, spore-forming and Gram-positive were retained.

The biochemical study based on the classic gallery with the following characters: respiratory type, catalase, nitrate reductase, indole production, fermentation of glucose and mannitol was performed by using galleries Api 50CHB (bioMérieux, Marcy the Etoile, France).

Search for parasporal crystal

This step is devoted to the highlighting of parasporal crystal which allows the distinction between *B. thuringiensis* and other species of the *B. cereus* group. The same smears prepared for observation under the optical microscope were used without staining for scanning electron microscope examination (SEM) Hitachi TM 100.

Molecular techniques

The presence of parasporal crystals is a diagnostic characteristic for Bt strains (Konecka et al., 2007). The taxonomic identity of the toxic crystalliferous isolates was confirmed by amplification and partial sequencing of their 16S rDNA genes (Alvarez et al., 2009a). DNA extraction was realized from 1 ml of culture incubated during night at 30°C in the medium (LB). The protocol described by Bravo et al. (1998) was used, followed by a universal 16S PCR targeting the gene coding for 16S rRNA using universal primers (Petiti, 2007). Indeed, ribosomal RNA (rRNA 16S) was the RNA component of the small ribosomal subunit of the prokaryotic 30S. The gene encoding this rRNA is the "16S rRNAgene" (Clarridge III, 2004), present in the whole bacterial species, in a variable number of copies (Petti, 2007; Woese, 1987). It consists of 1500 nucleotides and contains seven conserved regions and nine hypervariable regions (Chakravorty et al., 2007).

PCR conditions

The concentration of DNA used for PCR is between 10 and 30 ng/µL (DNA concentration is determined using a spectrophotometer (Nanodrop ™ 1000). The PCR program includes an initial denaturation step of 3 min at 94°C, followed by 30 cycles with a denaturation of 1 min, a hybridization of 30 s at 45°C, followed by an extension of 2 min at 72°C for the primer pair, and finally a final elongation of 10 min at 72°C. The reactive mixture consists of: 0.3 µL of each 16S primer, 2.5 µl of buffer containing 20 mM of $MgCl_2$, 0.2 µl (25 mM) of dNTPs, 0.2 µl of Taq polymerase, 20.5 µl of water and 1 µL of DNA. After that, 5 µl of the PCR amplified product, mixed with bromophenol blue was analyzed by electrophoresis on agarose gel (1%) after fixation with ethidium bromide. The gel was visualized with gel documentation system. Using a size marker, only the PCR products with a bands size corresponding to 1500 pb are selected for sequencing (Table 2).

Cloning and sequencing

The retained PCR products were purified, cloned and sequenced. The samples were prepared according to the recommendations of the laboratory. The obtained sequences were compared by basic local alignement search tools (BLAST) software set on line by the National Center for Biotechnology Information (NCBI) (USA).

Table 2. Sequences of the 16S rRNA genes primers used for PCR amplification.

Primer	Sequence (5' to 3')	Annealing temperature (°C)	Size (bp)
16S rRNA-F	AGAGTTTGATGCTCAG	56	1500
16S rRNA-R	CTACGGCTACTTACGA	56	1500

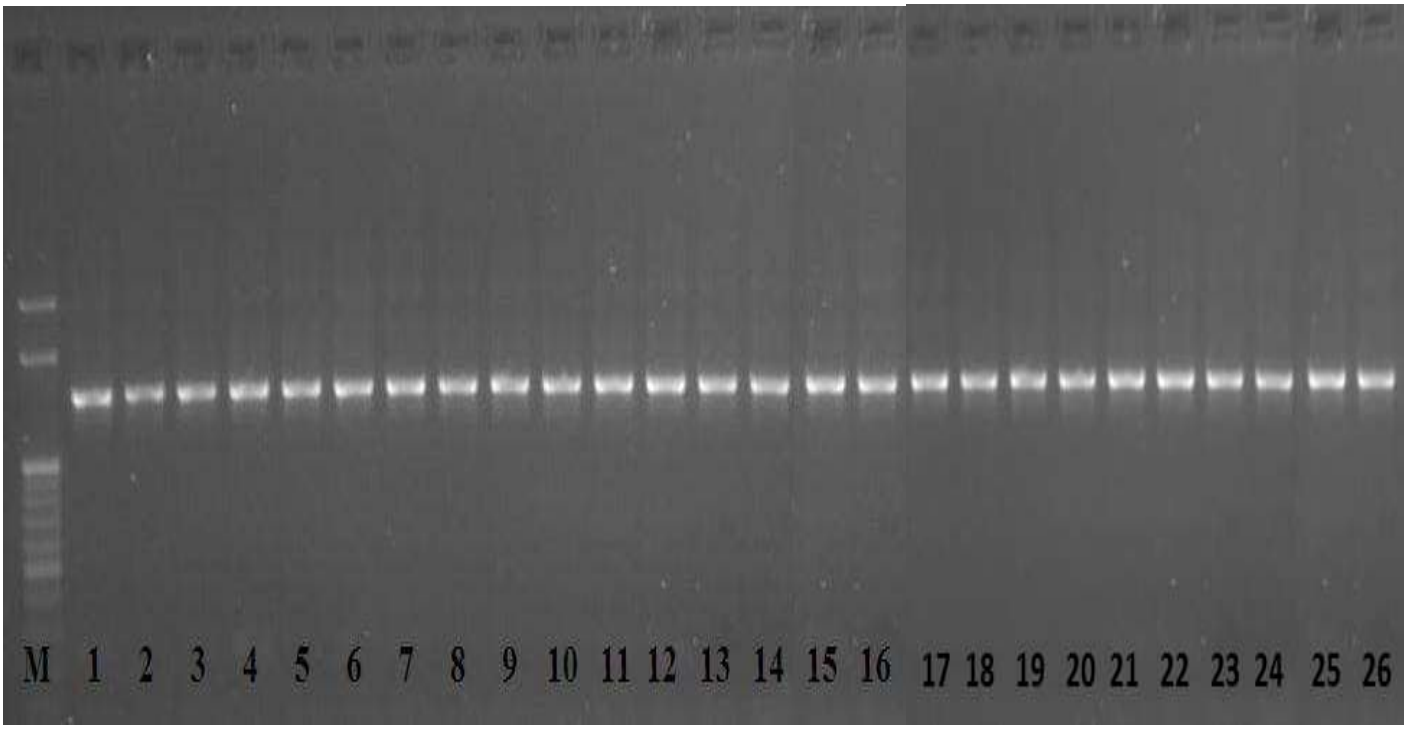

Figure 1. Agarose gel (1%) electrophoresis for 16s rRNA –PCR. Lane M: DNA MW marker 1 Kb, Lane 1: *B. thuringiensis* HD22, Lane 2: *B. thrungiensis* BMG7, Lane 3: S6DSP, Lane 4: S6DSG, Lane 5: S3DSG, Lane 6: S2DSP, Lane 7: S2DSG, Lane 8: BSUBG, Lane 9: BM5, Lane 10: BM3, Lane 11: BM14, Lane 12: BM13, Lane 13: BIS, Lane 14: BEN3, Lane 15: BEN1, Lane 16: B9, Lane 17: B8, Lane 18: B7, Lane 19: B6, Lane 20: B5, Lane 21: B4F, Lane 22: B3, Lane 23: B2C, Lane 24: B10, Lane 25: B1, Lane 26: 10).

RESULTS

Strain identification

The macroscopic characterization allowed distinguishing round colonies; flat (raised) with regular edges or not, opaque and white or beige.

The microscopic study, with Gram stain in fresh samples allowed us to notice that all strains are in the form of variable-sized rods (fine, medium and large); right with rounded edges and are Gram-positive, spore-forming and mobile. For the physiological tests, the result tends to show that all strains possess an optional catalase aero-anaerobic. The mini biochemical profile built on the basis of 50 tests of the API 50CHB gallery allowed one to distinguish a major group of 24 strains of various orchards (6 out of Tlemcen city, 5 of Oran city, 11 from Ain Defla city and finally 2 out of Sétif city). These strains present the largest number of positive characters, typical of species belonging to the *B. cereus* group. The analysis of these results by the Web API software gave the following identifications.

Observation by scanning electron microscopy

The strains observed by scanning electron microscope revealed that they belong to the group of *B. cereus* by the presence of parasporal crystal which is present only in *B. thuringiensis* (Figure 1), whereas this body is absent in the other bacteria of the group. The pictures taken by a scanning electron microscope show the presence of the crystal in 24 of our samples. The isolated Bt strains present parasporal crystals of cubic and rhombohedral shapes (Tables 3 and 4).

When the morphology of the crystals was examined,

Table 3. Crystal protein morphology of native isolates of *B. thuringiensis.*

Isolate	Shape of crystal protein
S6DSP	Cubical
S6DSG	Rhombohedral
S3DSG	Rhombohedral
S2DSP	Rhombohedral
S2DSG	Cubical
BSUBG	Cubical
BM5	Cubical
BM3	Cubical
BM14	Cubical
BM13	Cubical
BIS	Cubical
BEN3	Cubical
BEN1	Cubical
B9	Cubical
B8	Rhombohedral
B8	Rhombohedral
B7	Cubical
B6	Rhombohedral
B5	Rhombohedral
B4F	Cubical
B3	Cubical
B2C	Rhombohedral
B10	Cubical
B1	Rhombohedral
10	Rhombohedral

crystalline bipiramidal forms were determined by scanning electron microscopy after purification of the crystals by a special ultracentrifugation procedure (Fast, 1972).

Electrophoresis of PCR products

The analysis of PCR products by electrophoresis on agarose gel (1%) appears as shown in Figures 2 and 3.

DISCUSSION

Soil is a very important source of *B. thuringiensis* strain providing a large genetic resource for its use in the development of bio-insecticide to control insect pests that have not been previously reported to be susceptible to *B. thuringiensis* (Quesada-Morage et al., 2004).

On the bases of the dyeing properties, the production of endospore and its position, the mobility of strains, the presence of catalase and the absence of indole production, the strains that were isolated from the experimental orchards are assigned to the *B. cereus* group, which is the group I of the classification of Gordon et al. (1973).

The analyses by APIWEB software of API50 CHB galleries results have confirmed that all the 24 strains obtained belong to *B. cereus* group with species identification for 14 of them. Starting from the universal characteristic in the bacterial world, the amplification followed by sequencing of the 16S rRNA gene was used to identify the badly identified bacterial strains by some conventional techniques (Petti, 2007). The result of PCR 16S corresponds to the identifications with API50 CHB gallery, and presents huge similarities with these results using the indicated on-line software.

The findings are supported by those obtained by Guinebretière et al. (2001), in terms of our strains and reference strains, but did not allow us to distinguish *B. cereus* from *B. thuringiensis*. These results confirm the observation made by Abderrahmani (2011) concerning the use of RNA 16S for the identification of bacteria belonging to the cereus group.

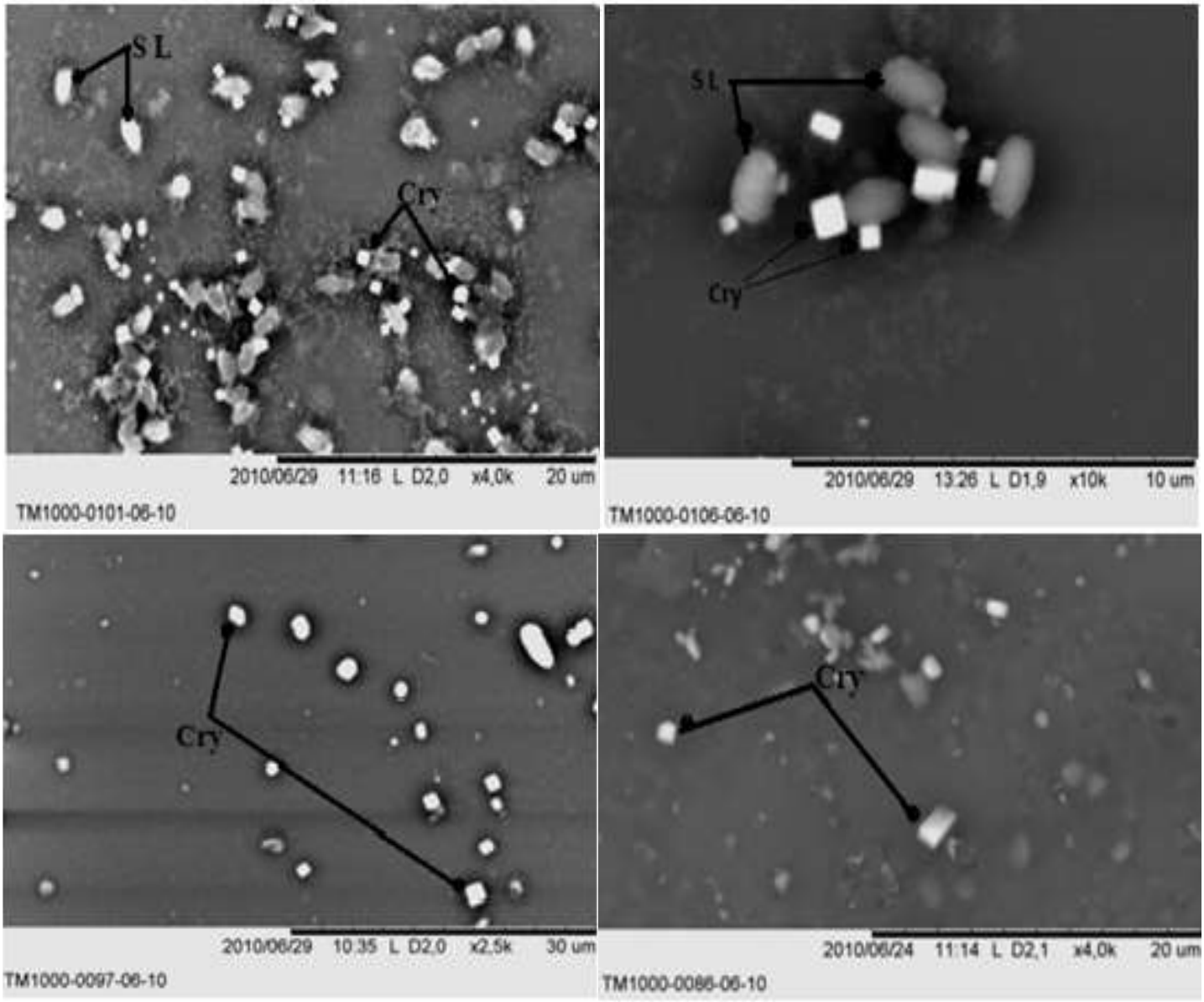

Figure 2. Photos taken by scanning electron microscope (SEM) (Cry: crystals and SL: free spores).

The authors could not discriminate between the two species until after the observation by scanning electron microscope, which allowed the visualization of the parasporal crystal which is present only in *B. thuringiensis* and responsible for entomopathogenic activity of this bacterium against several devastating species. The toxicity of *B. thuringiensis* did not depend on Cry gene content only because factors other than Cry proteins may contribute to toxicity as well as spore interaction with crystal protein and the other soluble toxins such as β-exotoxin (Porcar et al., 2000).

Martinez et al. (2004) suggested that the biological activity of a strain cannot be fully predicted on the basis of its Cry gene content alone. The relative proportion of the Cry proteins produced, their interaction, and the possible presence of undetected crystal proteins, such as cry1I genes are all important. These results show the diversity of the bacterial flora of *Bacillus* species in the Northern Algerian soil and the predominant presence of *B. thuringiensis* strain in the study areas, which could be considered as a potential reservoir of species used as a bio-pesticides for controlling insects pests without harming the beneficial ones.

Conclusion

This work confirmed that the bacterium, *B. thuringiensis*

Table 4. Treatment of the sequences obtained after the PCR16S by the BLAST tool of the NCBI.

Strain	Homology (%)	Closest relative	Acc. N.	Phylogenetic group
S6DSP	99	*Bacillus pumilus*	KJ526885.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
S6DSG	99	*Bacillus thuringiensis*	KJ011876.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
S3DSG	100	*Bacillus mycoides*	EU924505.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
S2DSP	99	*Bacillus subtilis*	KU821696.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
S2DSG	99	*Bacillus thuringiensis*	KJ937088.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
BSUBG	99	*Bacillus subtilis*	JQ361065.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
BM5	99	*Bacillus subtilis*	HM590703.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
BM3	99	*Bacillus thuringiensis*	HM068889.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
BM14	100	*Bacillus subtilis*	KM492825.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
BEN13	99	*Bacillus thuringiensis*	KM280648.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
BIS	99	*Bacillus thuringiensis*	KJ123714.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
BEN3	100	*Bacillus thuringiensis*	KJ769222.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
BEN1	100	*Bacillus thuringiensis*	CP010089.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
B9	99	*Bacillus cereus*	EU240373.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
B8	99	*Bacillus cereus*	KF758385.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
B7	99	*Bacillus thuringiensis*	KJ722441.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
B6	99	*Bacillus mojavensis*	KF600759.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
B5	99	*Bacillus thuringiensis*	KJ769221.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
B4F	99	*Bacillus thuringiensis*	CP010089.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
B3	100	*Bacillus thuringiensis*	CP010089.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
B2C	99	*Bacillus subtilis*	KM492825.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
B10	100	*Bacillus cereus*	EU240373.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
B1	100	*Bacillus thuringiensis*	KJ769218.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.
10	99	*Bacillus cereus*	EU240373.1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.

occurs naturally in Northern Algeria. This shows the possibility of using natural bio-insecticides, based on the local strains of *B. thuringiensis* which are against the harmful stages of the pests due to the various types oftoxins synthesized by this bacterium. The authors hope to continue this work with the aim to market the local strains of *B. thuringiensis* identified and put at the disposal of farmers. Indeed, in Algeria, the fight against fruit fly is based essentially on the use of insecticides.

Conflict of Interests

The authors have not declared any conflict of interests.

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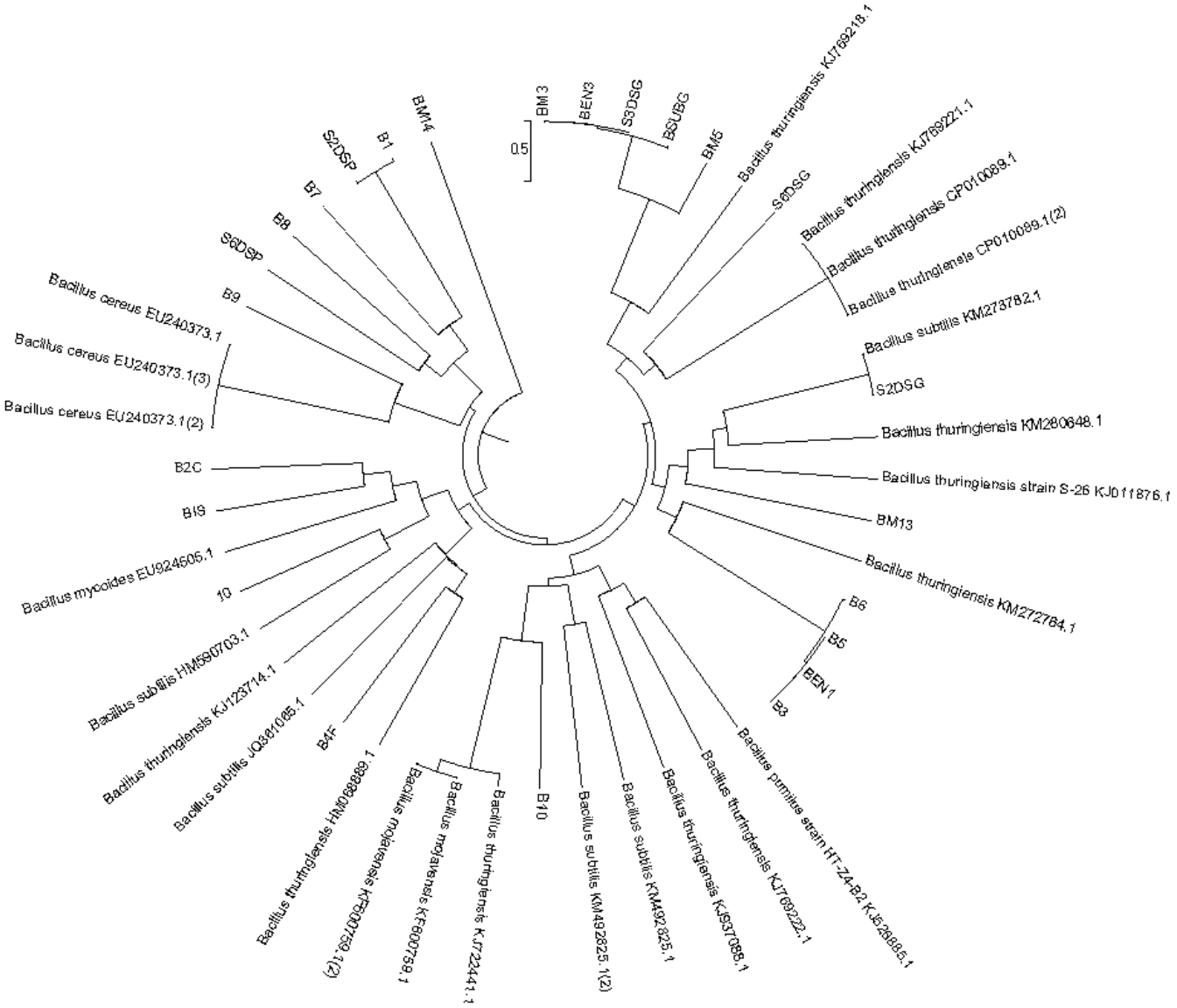

Figure 3. Dendrogram comparing PCR products of the field isolates and of the standard strains (DNA samples amplified in the presence of primers 16S-F and 16S-R).

Thabet resources (Tunisia), to enable us to realize the studies in his laboratory.

REFERENCES

Abderrahmani A (2011). Identification of non-ribosomal synthesis mechanism of a new lipopeptide kurstakine and the study of its influence on the strains of *Bacillus thuringiensis* isolated phenotype in Algeria. Thesis of State University of Science and Technology Houari Boumediene, Algiers, Algeria. P 87.

AFNOR (1996). Standard Method for microbiological analysis of food products.

Ben-Dov E, Zaritski A, Dahan E, Barak Z, Sinai R, Manasherob R, Khamraeb A, Troitskaya E, Dubitsky A, Berezina N, Margalith Y (1997). Extended screening by PCR for seven cry-group genes from field-collected strains of *Bacillus thuringiensis*. Appl. Environ. Microbiol. 63:4883-4890.

Bravo A, Sarabia S, Lopez L, Ontiveros H, Abarca C, Ortiz A, Ortiz M, Lina L, Villalobos FJ, Pena G, Nunez-Valdez, Soberon M, Quintero R (1998). Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. Appl. Environ. Microbiol. 64:4965-4972.

Bravo A, Gill S, Soberón M (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon. 49:423-435.

Brar S, Verma M, Tyagi R, Surampalli R, Bernabé S, Valéro J (2007). *Bacillus thuringiensis* proteases: Production and rôle in growth, sporulation and synergism. Process. Biochem. J. 42:773-790.

Carozzi N, Kramer V, Warren G, Evola S, Koziel MG (1991). Prediction of chain reaction product profiles. Appl. Environ. Microbiol. 57:3057-3061.

Chakravorty S, Helb D, Burday Mr, Connell N, Alland D (2007). Characterization of cry genes in a Mexican *Bacillus thuringiensis* strain collection. Appl. Environ. Microbiol. 64(12):4965-4972.

Clarridge JE III (2004). 16S rRNA Impact of Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. Clin. Microbiol. Rev. 17:840-862.
Fast PG (1972). The delta endotoxin of *Bacillus thuringiensis* III: a rapid method for separating parasporal bodies from spores. J. Invertebr. Pathol. 20:189-140.
Gordon RE (1973). The taxonomy of soil bacteria.In Gray and Parkinson (Editors). pp. 293-321.
Hansen B, Damgaard P, Eilenberg J, Pedersen JC (1998). Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated from leaves and insects. J. Invertebr. Pathol. 71:106-114.
Konecka E, Kaznowski A, Ziemnicka J, Ziemnicki K (2007). Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated during epizootics in *Cydia pomonella* L. J. Invertebr. Pathol. 94:56-63.
Martinez C, Porcar M, Lopez A, de Escudero FJ, Perez-Llarena IR, Caballero P (2004). characterization of a *Bacillus thuringiensis* strain with a broad spectrum of activity against lepidopteran insect. Entomol. Exp. Appl. 111:71-77.
Ongena M, Jacques P (2007). Bacillus lipopeptides: Versatile weapons for plant disease biocontrol. Trends. Microbiol. 16:116-125.
Padidam M (1992). The insecticid al crystal protein Cry1A (c) from *Bacillus thuringiensis* is highly toxic for Heliothis Armigera. J. Invertebr. Pathol. 59:109-111.
Petti CA (2007). Detection and identification of microorganisms by amplification and gene sequencing. Clin. Infect. Dis. 44:1108.
Porcar M, Martinéz C, and Caballero, P (2000). Host range and gene contents of *Bacillus thuringiensis* strains toxic towards Spodoptera exigua. Entomol. Exp. Appl. 97:339-346.
Quesada-Moraga E, Garcia-Tovar E, Valverde-Garcia P, and Santiago-Alvarez C (2004). Isolation, geographi- cal diversity and insecticidal activity of *Bacillus thuringiensis* from soils in Spain. Microbiol. Res. 159:59-71.
Rice PJ, Arthur EL Barefoot, AC (2007). Advances in Pesticide Environmental Fate and Exposure Assessments. J. Agric. Food. Chem. 55:5367-5376.
Woese CR (1987). Bacterial evolution. Microbiol. Rev. 51:222-270.

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Full Length Research Paper

Escherichia coli O157, *Staphylococcus aureus* and coliforms in crude and processed bovine milk marketed

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Fifty-five (55) samples from five municipalities in the Recôncavo from Bahia region were analyzed between May and July 2015 to evaluate the sanitary quality and the presence of pathogens of crude and processed bovine milk samples. Psichrotrophic, mesophilic and thermophile rates and the count of *Escherichia coli*, *E. coli* O157: H7 *Staphylococcus aureus* and *Listeria monocytogenes* were done by microbiological methods rapid detection. There was a greater contamination and presence of pathogens in the raw milk when compared to processed milk. However, total coliforms were detected in 14.28%, *E. coli* in 7.14% and *E. coli* O157: H7 in 2.04% of processed milk. The enforcement by authorities against the illegal sale of raw milk and the monitoring of steps in milk production up to marketing should be mandatory.

Key words: Sanitary conditions, dairy industry, food safety.

INTRODUCTION

Although Brazilian law forbids selling of milk *in natura*, it is still common practice in Brazil, associated with cultural, regional and social factors (Bersot et al., 2010; Menezes et al., 2015).

Dairies use thermal processes that reduce initial microbial load of raw milk, such as pasteurization and Ultra High Temperature (UHT), to commercialize safer food with regard to sanitary hygiene and shelf time increase, bur deficient hygiene conditions during milking, handling and conservation are the main factors for decrease in milk quality produced in Brazil (Menezes et al., 2015), damaging the country´s economic development in milk production.

Such practice may be harmful to consumer´s health since milk is a good vector of pathogenic microorganisms which cause Food-Transmitted Diseases (FTD) (Claeys et al., 2013).

Pathogenic bacteria in food is a matter of global food security. The risk of human illness associated with raw products can best be predicted by monitoring potential sources of microbial contamination in the area during the collection, during processing and distribution or retail. Thus, rapid and accurate identification of pathogenic bacteria in food samples are important for food quality assurance and to find outbreaks of these bacteria (Bersot et al., 2010; Tamanini et al., 2011; Weschenfelder et al., 2016).

Further, several studies have revealed the bad quality

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of milk produced in Brazil (Tamanini et al., 2011; Pereira et al., 2013; Weschenfelder et al., 2016), affected not merely by prime matter used but by lack of post-processing health control, especially during bottling and packaging. Prevention against milk contamination during milking and storing is mandatory to reduce microbial multiplication and to produce quality milk products (Salvador, 2012).

Since milk is a high economic asset in Brazil (USDA, 2015; IBGE, 2016) and its intake may be a health risk for the population due to microbial multiplication, the aim of the current paper is to assesses raw and UHT milk quality sold in the towns of the Recôncavo from Bahia region. The study is a warning for authorities for drastic control on milk producing farms and in the region´s market.

MATERIALS AND METHODS

Sampling

Fifty-five bovine milk samples were retrieved and analyzed between May and June 2015: four samples of untreated milk and seven whole UHT milk samples of different trademarks, from each municipality. Samples were purchased in five municipalities in the Recôncavo da Bahia region, namely, Cruz das Almas, Sapeaçu, Governador Mangabeira, Muritibaand São Felipe. Physical integrity, airtightness, packaging and lots verified during UHT milk sample collection were the selection criteria.

Samples were maintained in isothermal boxes with recyclable ice and immediately transported to the Laboratory for Food and Water Analytic Investigation of Agrarian, Environmental and Biological Sciences of the Universidade Federal do Recôncavo da Bahia, Cruz das Almas BA Brazil.

Packages for raw and UHT milk were initially washed with water and neutral detergent, dried with white disposable paper, hygienized with ethanol 70% and homogenized (shaken 25 times). UHT milk samples were incubated at 35-37ºC for seven days, following Brazilian legislation (1997, 2001).

Microbiological analysis

Psichrotrophic, mesophyll and thermophile microorganisms were counted by pour plate technique in Plate Count Agar (PCA) medium (APHA 1.05463.0500). Initially it was used 1 ml of each milk sample and transferred into a sterile tube containing 9 ml of 0.1% peptone water to give a dilution of 10^{-1}. From this dilution serial dilutions were performed to obtain the dilution 10^{-5} . Further, 1 mL of each dilution was transferred to sterilized Petri plates with 25 mL agar previously combined and warmed at 43-45ºC. After homogenization and solidification, the plates were incubated in a at 7ºC for 10 days; 35ºC for 48 h; 50ºC for 48 h for psichrotrophic, mesophyll and thermophile microorganisms, respectively.

Coliforms were counted in medium HiCrome ECC Selective Agar Base–M1294 Himedia® (HiMedia Laboratories Pvt. Ltd., Mumbai-India.) (ISO 9001:2008). Methodology complied to manufacturer´s instructions. Colonies ranging between salmon and red color were typical colonies of total coliforms, while dark blue to violet revealed *Escherichia coli* colonies.

Staphylococcus aureus populations were calculated by fast method on plates (3M Company) Petrifilm™ (AOAC 2003.11), following manufacturer´s instructions. Colonies of a red-violet color were typical of *S. aureus.*

E. coli O157:H7 analysis initially comprised a warming stage; the samples were then added to the selective enrichment broth MTSB Novobiocin, MERCK™, and incubated at 35°C-37°C for 18-24 h (Ahmed and Shimamoto, 2014).

Species of *E. coli* O157:H7 was identified by fast immunological scanning Singlepath®- *E. coli* O157, MERCK™ (Merck KGaA, Darmstadt - German) (AOAC 010407), following manufacturer´s instructions. Test apparatus was incubated at room temperature and result was given 20 min after the sample was applied to the apparatus. A negative result for strain *E. coli* O157:H7 occurred when only a red line appeared within the control zone (C); result was positive when red line appeared in tests (T) and control (C).

Listeria monocytogenes was identified by fast kit Singlepath® L'mono, MERCK™ (1.04148.0001). Milk samples were previously added to Brain and Heart Infusion (BHI) broth (Merck KGaA, Darmstadt - German) and incubated at 29-30ºC for 24 h, for selective enrichment. The presence of *L. monocytogenes* in kits with red line in the test zone (T) and in the control zone (C) of the apparatus was positive, but negative when no line occurred in the test zone (T), although it appeared clearly in the control zone (C).

Mean number of colonies in all plates was multiplied by the respective dilution factor and results were given in log CFU/ml (Brasil, 2003). Moreover, statistical analysis was undertaken by SPSS 17. Averages and standard deviation of the microorganisms were calculated with descriptive analysis.

Statistical analysis

Means were compared by Student´s *t* test for independent samples and evaluated whether there were any differences in the quantity of microorganisms according to type of milk. Pearson χ^2 test was employed to analyze qualitative categories, whilst rates $p \leq 0.05$ were significant.

RESULTS AND DISCUSSION

Since the commercialization of raw milk is illegal, Brazilian legislation has not provided parameters for the product. When Norm 62 published on 29 December 2011 (Brasil, 2011) on refrigerated raw milk for processing is applied, it has been found that 80.95% of samples revealed mesophyll microorganisms, of which 76.47% were above the rate allowed by current legislation, or rather, 5.87 log CFU/mL (Brasil, 2011). Samples from three out of the five towns averaged above the acceptable rate (Figure 1).

High population of mesophyll microorganisms in the raw milk analyzed may be attributed to inadequate sanitary conditions during milking and mainly to lack of refrigeration in transport, storage and commercialization of the product. It was perceived during collection of samples that most samples lacked refrigeration and directly contributed towards the proliferation of mesophyll microorganisms since best temperature for growth was that of room temperature.

Silva et al. (2010) evaluated the physical, chemical and microbiological quality of raw milk of a dairy farm in the state of Rio Grande do Suland reported that three out of the six milk samples analyzed were above legal rates (Brasil, 2011) for mesophyll microorganisms. Similarly to current study, Silveira and Bertagnolli (2014) analyzed

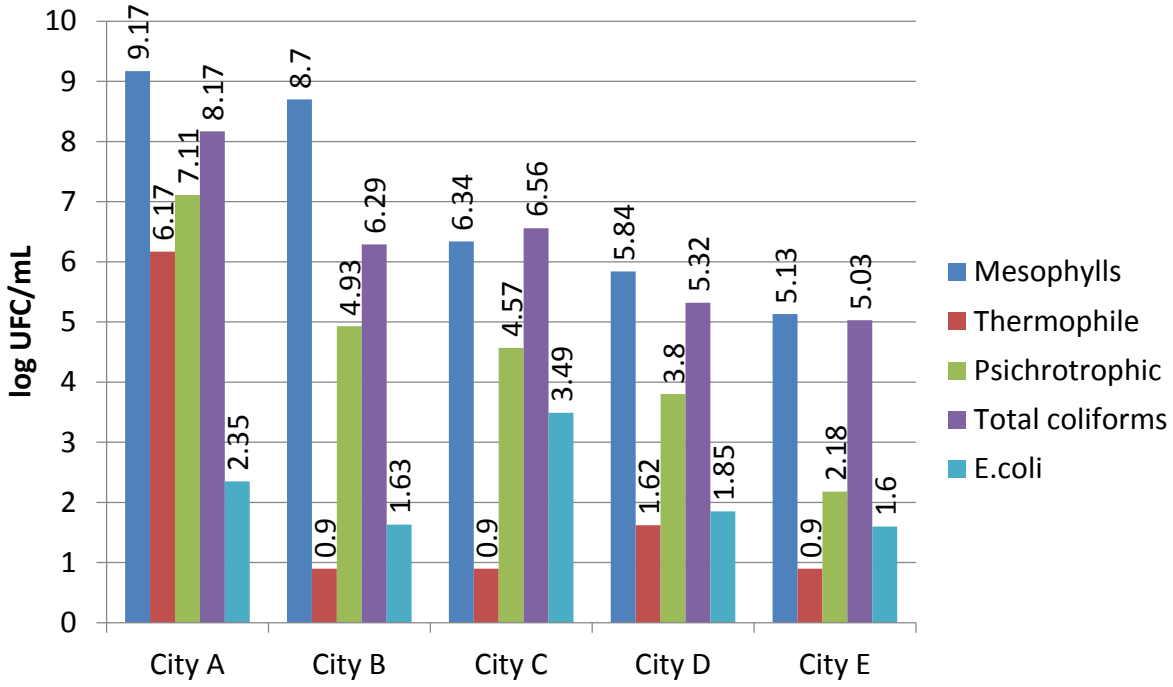

Figure 1. Means of microbial populations in milk samples in natura traded informally in the 2015 Bahia Reconcavo cities.

the quality of raw milk commercialized illegally in the street fairs of Santa Maria-RS Brazil and detected that three out of ten samples failed to comply with Norm 51 of 20/09/2002 for mesophyll organisms in refrigerated milk.

On the other hand, samples of UHT milk complied with Rule 146 of 07/03/1996 with rates up to 2 log CFU/mL for mesophylls. Studies by Frata et al. (2014) provided similar results, or rather samples were within the maximum mesophyll limit for UHT milk. However, Domareski et al. (2010) assessed UHT milk commercialized in three Mercosur countries (Brazil, Argentina and Paraguay), respectively reported 37.5, 62.5 and 12.5% mesophyll aerobic bacteria in milk, and thus failing to comply with microbiological criteria for UHT milk.

Storage temperature and type and initial microbial load are parameters that contribute towards the proliferation of bacteria during the storage of raw milk even when submitted to low temperatures. Raw milk kept for long periods at low temperatures may reveal psichrotrophic microorganisms and their concentration in milk may be associated to conditions in which the milk was obtained.

Although Brazilian legislation on the subject (Brasil 2001, 2011) does not establish a maximum limit for psichrotrophic and thermophile microorganisms, the Rules for Industrial and Sanitary Inspection of Animal-derived Products (RIISPOA) (Brasil, 1980) determine that milk should have a maximum of 10% psichrotrophic and thermophile microorganisms with total mesophyll counts.

According to the above recommendation, only 9.52% of raw milk samples in current analysis had the best pattern for psichrotrophic microorganisms. Zeni et al. (2013) verified the occurrence of psichrotrophic and mesophyll microorganisms in raw milk in the production of UHT milk. Samples of refrigerated raw milk had mesophyll and psichrotrophic counts above 6 log CFU/mL, which will surely interfere in the quality and shelf life of the final product.

Although raw milk does not normally have high rates of thermophile microorganisms, current study reveals averages between 6.17 and 1.62 log CFU/mL respectively for municipalities A and D, with 14.28% of samples above 10% of mesophyll microorganisms, blatantly not complying with current legislation (Brasil, 1980). There was a microbial multiplication of <1 log CFU/mL in all UHT milk samples.

Further, 90.47% of samples had over 5 log CFU/mL of total coliforms in raw milk. Although there is no maximum limit for total coliforms in raw milk when current sanitary legislation is taken into account, the microbial load is high in the samples. In fact, microorganisms reveal flaws in hygiene control and the possible presence of pathogenic microorganisms.

Corroborating results in current analysis, Dias et al. (2015) assessed the hygiene and sanitary conditions of raw milk commercialized in outdoor markets in the north of the state of Piauí, Brazil, and found that the 16 samples analyzed were positive for total and thermotolerant coliforms.

In a study in Solânea in the state of Paraíba, Brazil,

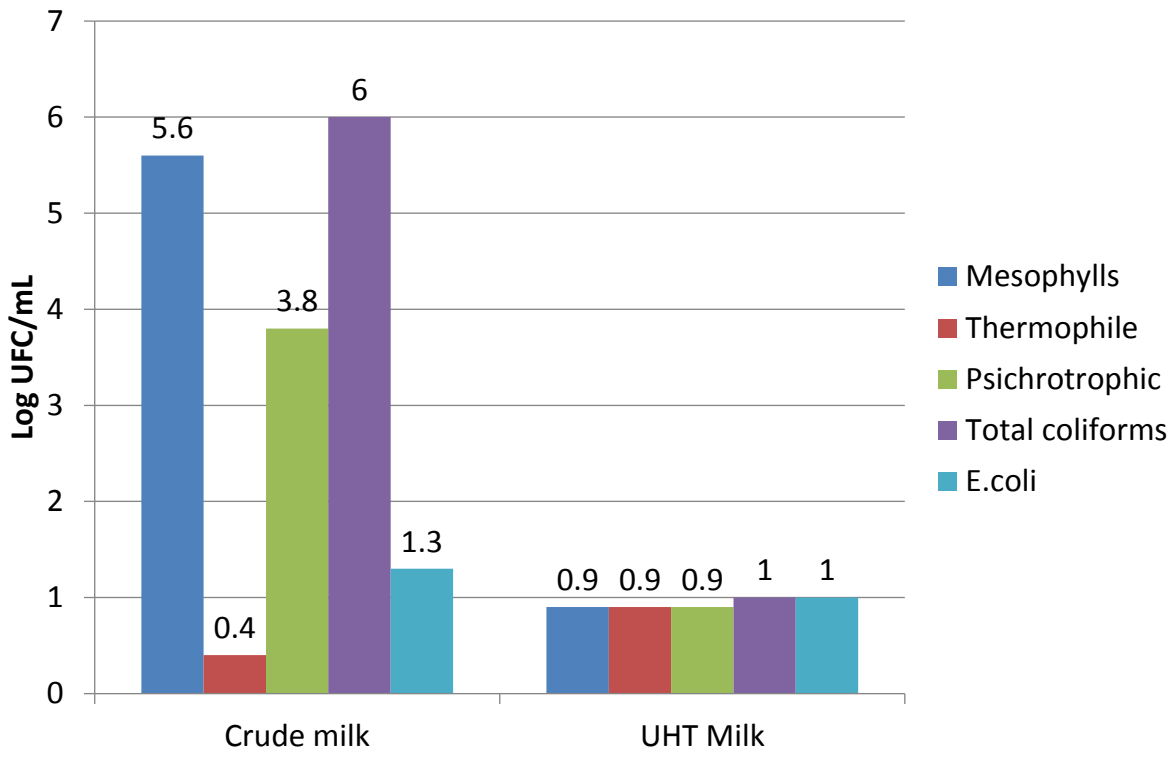

Figure 2. Compare the means of populations of microorganisms found in the types of milk.

Amaral and Santos (2011) reported that raw milk sold by street vendors had more than 1.100 most probable number (MPN/mL) of total and thermotolerant coliforms. Another study with similar results was performed by Maciel et al. (2008). The authors analyzed 30 samples of raw milk and detected total coliforms in all samples.

Samples from municipalities A and B showed average of 0.87 and 2.25 log CFU/mL of total coliforms in UHT milk, respectively, and thus not adequate for consumption. In fact, Resolution 370 of 4/9/1997rules that UHT milk should not contain microorganisms which are able to proliferate at normal storage and distribution conditions after the incubation of the closed package at 25-37ºC for seven days.

According to Menezes et al. (2015), hygienic milking procedures, cleansing and disinfection of utensils and equipments are basic to avoid milk contamination by coliforms. Training of milk producers with regard to hygiene at all stages in the provision and commercialization of raw milk is mandatory to guarantee the safeness of the product for industrialization.

E. coli was detected in samples of raw milk from all the municipalities and varied between <1 and 1.64 log CFU/mL, whereas it was detected in UHT milk from one municipality only, averaging 1.46 log CFU/mL. Its occurrence and its high population in milk are highly relevant for public health since, besides the existence of pathogenic strains, it is the main indicator for feces-caused contamination.

Microbiological analysis of raw milk revealed *S. aureus* in 76.19% of all samples analyzed, varying between <1 and 9.0 log CFU/mL. UHT milk samples had rates lower than <1 log CFU/mL. Student´s *t* test showed a statistical difference between raw and UHT milk, with higher contamination in the former ($p<0.05$).

Although there is no maximum limit for *S. aureus* in raw milk, its occurrence is associated with lack of hygiene of handlers´ hands, utensils used in milking, bad storage, transport and commercialization conditions. The above contamination situation was observed during sampling. In fact, milk could be found in inadequate containers, without any identification, featuring dirt rates. Others lacked refrigeration and this fact may have contributed towards the multiplication of the microorganism in most samples.

One may conclude that average counts for *S. aureus* populations in the milk from the municipalities were similar and showed that the presence and high population rates of *S. aureus* is normal in raw milk (Figure 2)

Research evaluating the microbiological quality of raw milk informally commercialized in Areia PB Brazil showed 80% of samples were contaminated by *Staphylococcus* sp (30% were presumably *S. aureus*) corroborating results in current study (Souza et al., 2011). Alves et al. (2009) assessed the microbiological quality of raw milk

commercialized in São Luís MA Brazil, and detected strains of *Staphylococcus* positive coagulase in 31.0% of samples.

Similar results were reported for UHT milk by Nascentes and Araújo (2012) who compared the microbiological quality of raw, pasteurized and UHT milk commercialized in Patos de Minas MG Brazil. The authors reported that all samples of raw milk were contaminated by *S. aureus*, whereas no growth of the microorganism was detected in UHT milk sample.

E. coli O157:H7 occurred in 6.12 and 2.04% of raw and UHT milk samples respectively, or rather, a potential risk for consumers since it is a pathogenic strain. Further, Pearson´s χ^2 test showed no significant difference between the types of milk studied with regard to *E. coli* O157:H7 ($p>0.05$). Since the microorganism is highly susceptible to heat, it may be easily destroyed during milk processing. However, post-processing contamination may occur due to its ability to form biofilms on utensils and equipments. According to Pillai and Jesudhasan (2006), the ability of *E. coli* O157:H7 in forming biofilms may be attributed to the self-induction-2 signal involving the regulation of genes in chemotaxis, flagellar synthesis and motility.

Batista et al. (2014) evaluated the hygiene and sanitary parameters of raw milk on 26 farms in the region of the Recôncavo da Bahia BA Brazil and reported one positive sample of *E. coli* O157:H7.

Above data show the importance of permanent studies on raw and processed milk, underscoring the need for the non-commercialization of raw milk directly to the consumer and the hygiene conditions during the post-processing stage.

It should be emphasized that *E. coli* O157:H7 in UHT milk is a serious factor due to the fact that milk had undergone commercial sterilization process and the consumer is liable to be contaminated by the pathogenic microorganism by purchasing an unsafe product.

In the case of *L. monocytogenes*, the result in all milk samples proved to be negative. In a study on *Listeria* spp. in raw and pasteurized milk, 10% of raw milk samples were contaminated by the microorganism, with no contamination of any pasteurized milk sample (Almeida et al., 2013).

Although current analysis did not reveal *L. monocytogenes*, its presence in milk is of great concern due to the microorganism´s high pathogenicity which causes listeriosis worldwide. Lack of detection of the pathogenic microorganism may also be related to its low capacity of competition by nutrients and thus undetectable, albeit present.

When all the aspects evaluated and results for microorganisms which indicate sanitary and pathogenic quality in raw milk in the municipalities under analysis are taken into account, the samples do not comply with current Brazilian legislation for refrigerated raw milk (Brasil, 2011) with regard to microbiological aspects. In fact, it is risk for public health and may be a FTD vector.

Good Practices in handling should be practiced by producers to reduce diseases caused by the incorrect handling of raw milk on the farms and in storage, transport and commercialization of the final product after a correct processing. Efficient measures for the eradication of the sale of raw milk should be aimed at by the health authorities since no commercialization of such a product should occur.

When the microbiological quality of UHT milk samples commercialized in the municipalities evaluated is assessed, several samples were not fit for consumption, possibly by post-processing microbial contamination by pathogens *E. coli* and *E. coli* O157:H7.

Since the microorganisms analyzed were not resistant to extreme temperatures such as in UHT milk processing, it may be suggested that samples with pathogenic microorganisms were contaminated after thermal processing either during packaging or due to biofilms on the equipments or deficient processing. According to Vittori et al. (2008), post-processing contamination may be due to deficiency in packaging sterilization or to recontamination by handlers. The emergence of biofilms in milk processing environments contributes towards an increase in the probability of microbial contamination of processed milk products with deteriorating and pathogenic microorganisms (Marchand et al., 2012).

Bad quality of raw milk used as prime matter and preservation problems during commercialization may also cause microbial contamination in processed milk with the possibility of milk deterioration prior to the recommended preservation period (Dey and Karim, 2013).

Dairy industries should guarantee the quality of the product by sanitary control during the whole processing period, from the acquisition of prime matter to sale.

Conclusions

1) The commercialization of raw milk is highly dangerous for consumers due to rates of pathogenic microorganisms found in it.
2) Contamination of UHT milk may have been due to flaws in post-processing sanitary control that favored strains of total coliforms, *E. coli* and *E. coli* O157:H7 in the commercialized milk.
3) Negative results occurred for *L. monocytogenes*in all samples under analysis.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Ahmed AM, Shimamoto T (2014). Isolation and molecular characterization of Salmonella enterica, *Escherichia coli* O157:H7

and Shigella spp. from meat and dairy products in Egypt. Int. J. Food Microbiol. 168-169:57-62.
Almeida VM, Pereira LS, Costa FN (2013). *Listeria* spp., coliformes, bactériasmesófilas e psicrotróficas no leite in natura e pasteurizadotipo C. Rev. Inst. Adolfo Lutz. 14-19:72.
Alves LMC, Amaral LA, Corrêa MR, Sales SS (2009). Qualidade microbiológica do leite cru e de queijo de coalho comercializados informalmente na cidade de São Luís – MA. Rev. Pesquisa em Foco. 17:2
Amaral CRS, Santos EP (2011). Leite cru comercializado na cidade de Solânea, PB: caracterizaçãofísico-química e microbiológica. Rev. Bras. de Produtos Agroindustriais. 13(1):7-13.
Batista AS, Barros LSS, Santos ESV, Silva MH (2014). *Escherichia coli* O 157: H7 em leite produzido no Brasil. Rev. Bras. Hig. San. An. 08(2):87-111.
Bersot LS, Daguer H, Maziero, MT, Pinto JPAN, Barcellos, V, Cunha.; Galvão, JA (2010) Raw milk trade: profile of the consumers and microbiological and physicochemical characterization of the product in Palotina-PR region. Revista do Instituto de Laticínios Cândido Tostes. 373:65:3-8.
Brasil (1980). Ministério de Agricultura e Abastecimento. Regulamento da Inspeção Industrial e Sanitária de Produtos de Origem Animal. RISPOA. Brasília. 166 p.
Brasil (1997). Ministério da Agricultura, Pecuária e Abastecimento. Portaria n° 370, de 04 de setembro de 1997. Regulamento Técnico para Fixação de Identidade e Qualidade do Leite U.H.T (U.A.T). Diário Oficial da República Federativa do Brasil, Brasília, 08 Sept. 1997.
Brasil (2001b) Ministério da Saúde. Agência Nacional de Vigilância Sanitária. Resolução RDC n.º 12, de 02 de janeiro de 2001. Diário Oficial da União**,** Brasília, DF**,** 30 de janeiro de 2001.
Brasil (2003). Ministério da Agricultura, Pecuária e Abastecimento. Departamento de Inspeção de Produtos de Origem Animal. Instrução Normativa n.°62, de 26 de agosto de 2003. Métodos analíticos oficiais para análises microbiológicas para controle de produtos de origem animal e água. Diário Oficial da República Federativa do Brasil, Brasília, DF, 18 Sept., 2003.
Brasil (2011). Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa nº 62, de 29 de dezembro de 2011. Aprova o Regulamento Técnico de Produção, Identidade e Qualidade do Leite tipo A, o Regulamento Técnico de Identidade e Qualidade de Leite Cru Refrigerado, o Regulamento Técnico de Identidade e Qualidade de Leite Pasteurizado e o Regulamento Técnico da Coleta de Leite Cru Refrigerado e seu Transporte a Granel, em conformidade com os Anexos desta Instrução Normativa. Diário Oficial da República Federativa do Brasil, Brasília, 31 dez. 2011.
Claeys WLS, Cardoen G, Daube J, Block K, Dewettinck K, Dierick L, Zutter A, Huyghebaert H, Imberechts P, Thiange Y, Vandenplas, Herman L (2013). Raw or heated cow consumption: Review of risks and benefits. Food Control Oxford. 251-262:31.
Dey S, Karim MH (2013). Study on physicochemical and microbial quality of available raw, pasteurized and UHT milk during preservation. Int. J. Sci. Invent. Today 2(2):150-157.
Dias JN, Fontinele LL, Machado SMO, Oliveira JS, Ferreira GP, Pereira ACTC (2001). Avaliação das condições higiênico-sanitárias de leite cru e queijo coalho comercializados em mercados públicos no norte do Piauí. Rev.Saúde e Pesquisa. 8(2):277-284.
Domareski JL, Bandiera NS, Sato RT, Aragon LC, Santana EHW (2010). Avaliação físico-química e microbiológica do leite UHT comercializa do em três países do Mercosul (Brasil, Argentina e Paraguai). Arch. Latino Am. Nutr. 60-3:261-269.
Frata MT, Bachi AJ, Oliveira JG, Schneider AE (2014). Estudo da Qualidade Microbiológica e Físico-Química de Leites UHT Comercializados no Município de Dois Vizinhos – PR. In: Proceedings of the XII Latin American Congress on Food Microbiology and Hygiene,1., 2014, São Paulo. Anais...São Paulo: Blucher Food Science Proceedings. pp. 247-248.
IBGE. Instituto Brasileiro de Geografia e Estatística. Indicadores IBGE: Estatística de produção pecuária, 2015.
Maciel JF, Carvalho EA, Santos LS, Araujo JB, Nunes VS (2008). Qualidade microbiológica de leite cru comercializado em Itapetinga-BA. Rev. Bras. de Saúde e Produção Animal. 443-448:09-03.
Marchand S. Vandriesche G, Coorevits A, Coudijzer K, De Jonghe V, Dewettinck K, De Vos P, Devreese B, Heyndrickx M, De Block J (2009). Heterogeneity of heat resistant proteases from milk Pseudomonas species. Int. J. Food Microbiol. 133(1):68-77
Menezes IR, Almeida AC, Morão RP, Reis SV, Santos CA, Lopes ILN (2015). Qualidade microbiológica do leite cru produzido no Norte de Minas Gerais. Rev. Bras de CiênciaVeterinária 22(1):58-63.
Nascentes RM, Araújo BC (2012). Comparação da qualidade microbiológica de leite cru, pasteurizado e UHT comercializados nacidade de Patos de Minas, MG. Perquirere. 9(1):212-223.
Pereira JR, Tamanini R, Rios EA Oliveira VHS, Yamamura AAM, Beloti VB (2013). Microbiota mesófila aeróbia contaminante do leite UHT. Rev. do Instituto de Laticínios Cândido Tostes. 68(394):25-31.
Pillai SD, Jesudhasan PR (2006). Quorum sensing: how bactéria communicate. Food Technol. 60:42-50.
Salvador FC, Burin AS, Frias AAT, Oliveira FS, Faila N (2012). Avaliação da qualidade microbiológica do leite pasteurizado comercializado em Apucarana-PR e Região. RevistaF@pciência. 9-5:30-41.
Silva VA, Rivas PM, Zanela MB, Pinto AT, Ribeiro MR, Silva FP, Machado M (2010). Avaliação da qualidade físico-química e microbiológica do leite cru, do leite pasteurizado tipo A e de pontos de contaminação de uma Granja Leiteira no RS. Acta scientiae Veterinariae. 38(1):51-57.
Silveira MLR, Bertagnolli SMM (2014). Avaliação da qualidade do leite cru comercializado informalmente em feiras livres no município de Santa Maria RS.Rev.VigilânciaSanitária e Debate. 2(2):75-80.
Souza FM, Nogueira MS, Nunes FC (2011). Qualidade microbiológica do leite cru comercializado informalmente na cidade de Areia-PB. AgropecuáriaTécnica. 32(1):168-171.
Tamanini R, Beloti V, Ribeiro Junior JC, Silva LCC, Yamada AK, Silva FA (2011) Contribuição ao estudo da qualidade microbiológica e físico-química do leite UHT. Rev. Inst. de Laticínios Cândido Tostes. 27-33:66-382.
USDA (2015). United States Department of Agriculture: Foreign Agricultural Service. Available at: http://www.fas.usda.gov/on January 2015.
Vittori J, Schocken-Iturrino RP, Poiatti ML, Pigatto CP, Chioda TP, Ribeiro CAM, Garcia G R, Ragazani AVF (2008). Qualidade microbiológica de leite UHT caprino: pesquisa de bactérias dos gêneros *Staphylococcus*, *Bacillus* e *Clostridium*. Ciencia Rural. 38(3).
Weschenfelder S, Paim MP, Gerhardt C, Wiest JM (2016). Avaliação da rotulagem nutricional e das características físico-químicas e microbiológicas de diferentes marcas de leite pasteurizado e leite UHT. Boletim de Indústria Anim. 73-1:32-38.
Zeni MT,de Souza Maran MH, rabaioli da Silva GP, de Carli EM, Palezi SC. (2013). Influência dos microrganismos psicrotróficos sobre a qualidade do leite refrigerado para produção de UHT. Unoesc&Ciência ACET 4(1):61-70.

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Full Length Research Paper

Rhizobia isolation and selection for serradella (*Ornithopus micranthus*) in Southern Brazil

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Serradella is a plant that belongs to the Fabaceae family, with recognized forage value and has the ability to associate symbiotically with rhizobia. The objective of this study was to isolate, authenticate and select effective rhizobia obtained from native serradella (*Ornithopus micranthus*) to reduce the need for mineral fertilizer. Nodules, roots of serradella and rhizosphere soil samples were collected in seven municipalities from Rio Grande do Sul and Santa Catarina states. Bacterial colonies were isolated and identified based on morphological characteristics. The authentification and initial selection of rhizobia were conducted *in vitro*. The most promising strains from the *in vitro* study were evaluated in a greenhouse experiment for 60 days. Among the 148 bacterial cultures characterized, 113 induced the formation of nodules in serradella while 32 isolate effectively increased fresh mass of plants under *in vitro* conditions. The strains UFRGS Om57, UFRGS Om59 and UFRGS Om148 formed nodules with greater dry mass and produced high dry mass of plants (shoots and roots) which allowed greater accumulation of N in the shoots.

Key words: Plant growth promoting rhizobacteria (PGPR); biological fixation of nitrogen; *Ornithopus micrantus*; pasture.

INTRODUCTION

The serradella [*Ornithopus micranthus* (Benth.) Arechavaleta] is a plant that belongs to the Fabaceae family, with recognized suitability for grazing by cattle. Its leaves are rich in protein, reaching the crude protein content close to 20 % in the vegetative period (Fernandes and Reis, 2001). According to Dartora et al. (2012), the supply of proteins through pastures is important to balance nutritionally pastures which will be offered to ruminants, reducing the need for concentrated feed.

The serradella is adapted to lowland areas which tolerates high moisture content in the soil, with good root

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system and excellent nodulation capacity in flood conditions (Menezes et al., 2001), taking place before the summer crop. Because of its ability to produce green mass in lowland areas, it is used in experimental crops as cover crops in crop rotation systems after the cultivation of rice. It also has great potential for intercropping with ryegrass and great capacity for survival and development in acid soils (Freixial and Barros, 2012), typical in the state of Rio Grande do Sul. It is recommended to make the cuts together with the flowering period, from which its quality has drastically reduced, while wet seasons allow a second cut (Fernandes and Reis, 2001).

In addition to offering green mass and protein, serradella offers nitrogen (N) supply to the soil-plant system. It is also capable of establishing symbiosis with rhizobia which perform biological fixation of atmospheric nitrogen (BFN) and therefore provide essential nutrient to animals and other plants that make up the ecosystem of the pasture.

In a study by Jandrey (2008), the production of dry matter of the aerial part of serradella was 2.2 t ha^{-1}, lower than that obtained with ryegrass but equivalent to birdsfoot trefoil. As for the nitrogen (N) accumulated in the shoots, it was observed by Jandrey (2008) that an average of 63.1 kg N. ha^{-1} accumulate the area where serradella has been grown. This quantity of N is equivalent to that obtained with birdsfoot trefoil and higher than that obtained with ryegrass, showing it efficiency in biological nitrogen fixation.

In the case of grasses cultivation in succession to serradella, the N content found in the shoot of the legume, as well as the N present in the nodes and roots, certainly favor the successor crops during vegetative growth. Other possible benefits of the succession of legume/grass crop system are the production of phytohormones and other plant stimulating substances by rhizobia symbionts with the species that inhabit the rhizosphere of crops that follow them (Yanni and Dazzo, 2010; Yanni et al., 2011).

Despite the great potential of serradella as native pasture in southern Brazil, there is little use of this forage legume in feeding cattle that produce meat or milk in the farms of Rio Grande do Sul. One of the important reasons is that, there are few studies about selection of nitrogen-fixing bacteria for this plant, which hinders the adaptation of it to new environments, and also affects the expression of its maximum productive potential. Currently, in Brazil there are strains of rhizobia such as SEMIA905 and SEMIA929 strains which are recommended for the kind of serradella *Ornithopus sativus*. However, there are no strains recommended for the *Ornithopus micranthus* species, which occurs in lowland areas in succession to rice cultivation. Thus, it is possible to increase the yield of *Ornithopus micranthus* through the selection and use of efficient rhizobia to hold the symbiosis with the legume, and through symbiotic association it provide increased N content attached to the soil-plant system, as well as increasing the green mass contribution of serradella in pasture farming systems.

Rhizobacteria that promote plant growth are inserted into pasture production systems for feeding cattle that produce meat or milk. The present mechanisms that promote plant growth may be recommended for the composition of commercial inoculants for specific crops. In the case of rhizobacteria present in pastures, plants yield increment mechanisms which anticipate the supply of pastures, lengthen the crop production cycle, or increase the supply of pastures to animals at the same time reduce the need for use of mineral fertilizers.

This study aimed to isolate, authenticate and select effective and efficient rhizobia as to the nitrogen fixation in symbiosis with native serradella of the *Ornithopus micranthus* species, which may increase the N content of the leaf tissue and provide a better yield culture.

MATERIALS AND METHODS

Obtainment of rhizobium nodules in serradella plants

Soil samples and serradella plant of the *Ornithopus micranthus* species were collected (*Ornithopus micranthus*) in southern towns Cachoeirinha, Palmeira das Missões, Passo Fundo, Porto Alegre, Santa Vitória do Palmar and São Martinho da Serra, as well as in Correia Pinto town, Santa Catarina. Samples were collected in pastures with the presence of serradella (Table 1). Soil samples were collected with cutting shovel at 0 to 15 cm depth in rhizospheric region of plants. In addition to soil samples, whole serradella plants were also collected, with shoot and root system. In the laboratory, the roots were separated and thoroughly washed with running water for subsequent posting of nodules. Nodules were highlighted, dried on paper towels and packed in glass jars with silica and cotton for preservation. In the greenhouse, to obtain nodules from soil samples, soil samples will be suspended in saline (NaCl 0.85 %). Then, with the aid of a sterile pipette, 5 ml of solution was inoculated in serradella plants (*Ornithopus micranthus*) grown in Leonard jars (Vincent, 1970) of 700 ml, containing a sterilized mixture of vermiculite and sand (2:1). Nutrients were added to plants through the nutrient solution Sarruge 25 % (Sarruge, 1975) without nitrogen, and then sterilized. For disinfection, the serradella seeds were immersed in alcohol (70 %) for 30 seconds, followed by sodium hypochlorite (2.5 %) for 30 seconds, wash for seven consecutive times with sterile distilled water by autoclaving at 120°C for 20 min, and then stored at room temperature (20 to 25°C) for 24 h of germination. Inoculation of soil samples suspensions was performed when plants were seven days with 1 to 3 pairs of leaves. The inoculation was performed under axenic conditions with automatic pipetter (Labmate brand), adding 2 ml of soil solution per pot. At 45 days after inoculation, plants were collected and each shoot was separated from the root system. After washing the roots, nodules obtained in the greenhouse were highlighted, dried on paper towels and placed in glass containers silica and cotton according to the methodology described by Beck et al. (1993).

Obtaining bacterial isolates

After storage period of 1 to 2 months, nodules were rehydrated in

Table 1. Phenotypic characterization of isolates obtained for serradela (*Ornithopus micrantus*). The parameter growth time (days) refers to the time between the inoculation on solid medium and the final evaluation. All colonies showed convex elevation and entire margin, so these features are not shown in the table.

Isolates groups*	Growth time (Days)	Color	Diameter (mm)	Consistency	Opacity	Form	Locations	Number of Isolates	REI (%) Average
Group 1	10	Milky	1 a 2	Gummy	Opaque	Circular	Cach./P. A./S.V.P.	37	109,19
Group 2	10	Milky	<1	Aqueous	Translucent	Punctiform	Cach./S.V.P.	30	117,17
Group 3	10	Milky	<1	Aqueous	Opaque	Punctiform	Cach./S.V.P.	33	198,64
Group 4	10	Milky	< 1	Gummy	Translucent	Punctiform	Cach./C.P.	8	236,25
Group 5	3 a 4	Pink	3 a 4	Gummy	Opaque	Circular	Cach./P.F.	10	42,00
Group 6	4	Milky	3 a 4	Aqueous	Opaque	Circular	Cach./C.P.	8	215,00
Group 7	10	Transparent	<1	Gummy	Opaque	Punctiform	Cach.	1	520,00
Group 8	10	Transparent	<1	Aqueous	Translucent	Punctiform	Cach.	5	285,00
Group 9	10	Pink	1 a 2	Aqueous	Opaque	Circular	P.M.	3	30,00
Group 10	3 a 4	Pink	3	Aqueous	Opaque	Circular	P.M.	1	15,00
Group 11	8 a 10	Pink	1 a 2	Gummy	Opaque	Circular	P.A./S.M.S.	4	60,00
Group 12	4	Pink	3 a 4	Viscous	Opaque	Circular	P.M.	2	85,00
Group 13	10	Milky	<1	Gummy	Opaque	Punctiform	C.P.	4	83,75
Group 14	10	Milky	<1	Viscous	Translucent	Punctiform	P.M.	1	120,00
Group 15	10	Milky	<1	Butter	Translucent	Circular	C.P.	1	320,00
-	-	-	-	-	-	-	-	148 (Total)	41,88

* Isolates groups with the same morphological characteristics; REI (%) Average: Relative efficiency index (%) for each isolates group, described in Experimental section; Abbreviations: Cach.: Cachoeirinha; C.P.: Correia Pinto; P.F.: Passo Fundo; P.A.: Porto Alegre; P.M.: Palmeira das Missões; S.V.P.: Santa Vitória do Palmar; S.M.S.: São Martinho da Serra.

sterile distilled water for 24 h and kept in the refrigerator for the next day. After rehydration, nodules were separately macerated in sterile test tube with a glass rod sterile in laminar flow hood. With the aid of sterile pipette tips in a laminar flow hood, macerated nodules broths were inoculated into standard petri dishes with 9 cm diameter, containing the yeast extract mannitol agar (YMA) medium which contain 0,0025 % (w/v) congo red (Vincent, 1970), using drop method (Miles & Misra, 1988) and scattering (Buck and Cleverdon, 1960), separately.

Petri dish containing, medium YMA inoculum was stored in an oven at 28°C. Daily evaluation was performed in each of the plates with the aid of a magnifying glass table. As colonies with morphological features of rhizobia emerged, they were immediately transferred to another plate in order to obtain pure colonies with persistent morphology. The isolates were characterized according to time of growth characteristics, color, colony high, colony diameter, colony consistency and opacity of the colony, according to Conn et al. (1957). After phenotypic characterization, each isolate was inoculated into three test tubes containing YMA medium (Vincent, 1970). These inoculated tubes were stored in the Rhizobia Culture Collection of UFRGS.

Authentication and initial selection of rhizobia experiment

The isolates obtained in the previous step were evaluated for the ability to induce the formation of nodules and promote the growth in serradella plants. For this, an *in vitro* experiment was conducted in the laboratory, using 30 ml test tube containing a paper towel strip and 15 ml of nutrient solution Sarruge 25 % (Sarruge, 1975) without nitrogen and sterilized by autoclaving at 120°C for 20 min. For production of culture broths, the rhizobia were inoculated in test tubes with screw containing liquid yeast extract mannitol culture medium (YM) (Vincent, 1970) and placed in an orbital incubator at 28°C with shaking at 120 rpm for seven days. On the day of inoculation, the broth had a cell concentration of 10^8 colony forming units per ml (ufc mL^{-1}), determined in a Neubauer chamber (Moura et al., 1987).

The serradella seeds were sterilized by successive immersions in alcohol (70 %) for 30 seconds, followed by sodium hypochlorite (2.5%) for 30 seconds and wash for seven consecutive times with sterile distilled water.

Then, for pre-germination, the seeds were sterilized and placed on moistened paper towels with sterile distilled water, maintained at 22°C for 24 h. In laminar flow chamber, each 30ml tube received a disinfected pre-germinated serradella seed, with the aid of a clamp fowl. One day after seedling emergence, the isolates were inoculated individually in a laminar flow hood. Each 30 ml tube with a serradella plant received 1 ml of culture broth with an isolate. After inoculation the tubes were stoppered with cotton plugs and kept in lamps for 8 h of light per day in an average ambient temperature of 24°C.

The experiment consist of 150 treatments with three replications arranged in a randomized design. As for the treatments, 148 were composed of isolates from different colonies. Two not-inoculated control treatments were also conducted. One of the Control treatments received an addition of mineral N (Control + N), with an aliquot of 107 µl ml of NH_4NO_3 solution (20 g L^{-1}) equivalent to application of 100 kg of N ha^{-1}. The other control without inoculation did not receive the addition of mineral N (Control - N). After a period of 35 days, the experiment was terminated and the plants were collected. The plant fresh mass, number of leaves, number of nodules and length of shoot and root were quantified. For each inoculated treatment, relative efficiency index (REI) was used to estimate the bacterial efficiency on symbiotic nitrogen fixation (Brockwell, 1966). The REI was measured with the following formula:

$$REI\ (\%) = ((\ FM\ Inoculated) - (FM\ Control\ N\ -)) / ((FM\ Control\ N\ +) - (FM\ Control\ N))x100$$

Where:
FM Inoculated = Plant fresh mass of inoculated treatment
FM Control N - = Plant fresh mass of non-inoculated treatment without the addition of nitrogen
FM Control N + = Plant fresh mass of inoculated treatment with the addition of nitrogen equivalent to 100 kg N ha^{-1}.

Evaluation of symbiotic efficiency of rhizobia in serradella plants

Among the isolates that produced promising results *in vitro* environment, eight were chosen for symbiotic efficiency test in the greenhouse. Besides these, we also tested the two strains currently recommended in Brazil to serradella. In Brazil, there is no isolated released for *Ornithopus micranthus* species, we use SEMIA 905 (*Bradyrhizobium japonicum*) and SEMIA 929 strains (*Bradyrhizobium japonicum*), released by the Ministry of Agriculture, Livestock and Food Supply (MAPA) for the production of inoculants for the species of serradella *Ornithopus sativus*, obtained from the Rhizobia Cultures Collection of the Agricultural Research Foundation of Rio Grande do Sul (FEPAGRO).

Inoculations were made in Leonard jars (Vincent, 1970) of 700 ml, containing a mixture of vermiculite and sand in the ratio 2:1, at the top and bottom part of the nutrient solution without nitrogen (Sarruge, 1975).

The inoculations were performed using aliquots of 2 ml broth cultures, with about 10^8 ufc ml^{-1}, of each studied Rhizobium, grown in yeast-mannitol medium for seven days at 28°C, 120 rpm. The experiment consisted of; two control treatments without inoculation, one without nitrogen addition and the other with addition of two aliquots of 5.4 ml of NH_4NO_3 (20 g L^{-1}) solution equivalent to the addition of 250 kg of N ha^{-1}. The ten inoculated treatments were composed by the inoculations of isolates UFRGS OM4, UFRGS Om27, UFRGS Om57, UFRGS Om59, UFRGS Om62, UFRGS Om67, UFRGS Om82, UFRGS Om148, SEMIA 905 and SEMIA 929. Four replications per treatment were performed, arranged in a completely randomized design. The serradella seeds were sterilized and pre-germinated as described in the previous section. At the end of 60 days after emergence of the plants, the shoot was separated from the root system, wrapped in paper bags and subjected to drying in an oven at 65°C to constant weight for 96 h. The shoot was weighed after drying and then grounded for chemical determination of nitrogen accumulation in tissue according to the methodology described by Tedesco et al. (1995). The nodules were detached from the roots, counted and placed in an oven at 65°C for drying and dry weight determination at 48 h. The data obtained were submitted to analysis of variance and average medium test (Scott Knott, 5%), using the statistical program SISVAR (Ferreira, 2000).

The relative efficiency index (REI) of nitrogen fixation of the isolates (Brockwell et al., 1966) was calculated using the formula below:

$$REI = ((Ntotal\ trat. - Ntotal\ T\text{-}N) / (Ntotal\ T+N - Ntotal\ T\text{-}N))x\ 100$$

where:
N total trat = Total nitrogen of the inoculated treatment plant
N total T-N = total nitrogen of the uninoculated and without nitrogen control
N total T+N = total nitrogen of the uninoculated control and that received nitrogen supplementation.

RESULTS

Morphological characterization, authentication and isolated efficiency test

With collection and isolation studies, we obtained 148 isolates with typical bacterial characteristics. The isolates were grouped into 15 different groups, based on colonies morphological characteristics (Table 1). In addition, all serradella isolates obtained in this study showed concave colonies and regular edges.

The composition of the groups is shown in table 2. The groups with the highest number of isolates were; groups 1, 3 and 2 with 37, 33 and 30 isolates respectively. The Relative efficiency index (REI %) average was 41, 88 %. The REI (%) ranges from 15 and 30 % in Group 9 and 10 formed by inefficient isolate up to 320 % in Group 15 (Table 1). The best REI (%) were obtained with an inoculation of isolated UFRGS Om62 and UFRGS Om9, with values above 750%. Figure 1 shows the morphological similarity dendrogram of an isolates group. The lowest similarity observed was 75 % with a comparison of groups 5, 6, 10 and 12 with each other groups. This represents a high similarity between the groups. Of the total isolates, 127 had smaller colonies than 2 mm in eight to ten days of growth, classified in this study as slow-growing colonies. This slow growth accompanied by the formation of small colonies is also presented by strains SEMIA905 and SEMIA929, recommended for inoculant composition for serradella (*Ornithopus sativus*) and belonging to the genus *Bradyrhizobium*.

Moreover, the other 21 isolates were observed with

Table 2. Groups of isolates with identical morphological characteristics.

Group	Isolates
Group 1	UFRGS Om1, UFRGS Om4, UFRGS Om13, UFRGS Om14, UFRGS Om15, UFRGS Om19, UFRGS Om22, UFRGS Om23, UFRGS Om24, UFRGS Om25, UFRGS Om26, UFRGS Om27, UFRGS Om28, UFRGS Om29, UFRGS Om32, UFRGS Om37, UFRGS Om40, UFRGS Om42, UFRGS Om44, UFRGS Om45, UFRGS Om46, UFRGS Om51, UFRGS Om73, UFRGS Om75, UFRGS Om88, UFRGS Om89, UFRGS Om90, UFRGS Om91, UFRGS Om92, UFRGS Om93, UFRGS Om94, UFRGS Om95, UFRGS Om96, UFRGS Om97, UFRGS Om98, UFRGS Om104, UFRGS Om140
Group 2	UFRGS Om2, UFRGS Om3, UFRGS Om16, UFRGS Om20, UFRGS Om21, UFRGS Om43, UFRGS Om47, UFRGS Om48, UFRGS Om49, UFRGS Om52, UFRGS Om76, UFRGS Om77, UFRGS Om85, UFRGS Om86, UFRGS Om114, UFRGS Om115, UFRGS Om116, UFRGS Om117, UFRGS Om118, UFRGS Om119, UFRGS Om120, UFRGS Om121, UFRGS Om122, UFRGS Om123, UFRGS Om124, UFRGS Om126, UFRGS Om127, UFRGS Om128, UFRGS Om138, UFRGS Om142
Group 3	UFRGS Om6, UFRGS Om7, UFRGS Om9, UFRGS Om10, UFRGS Om11, UFRGS Om12, UFRGS Om50, UFRGS Om54, UFRGS Om55, UFRGS Om56, UFRGS Om58, UFRGS Om62, UFRGS Om63, UFRGS Om64, UFRGS Om65, UFRGS Om66, UFRGS Om67, UFRGS Om68, UFRGS Om70, UFRGS Om71, UFRGS Om72, UFRGS Om79, UFRGS Om81, UFRGS Om84, UFRGS Om87, UFRGS Om105, UFRGS Om106, UFRGS Om108, UFRGS Om109, UFRGS Om110, UFRGS Om111, UFRGS Om112, UFRGS Om143
Group 4	UFRGS Om17, UFRGS Om18, UFRGS Om38, UFRGS Om41, UFRGS Om57, UFRGS Om74, UFRGS Om136, UFRGS Om148
Group 5	UFRGS Om35, UFRGS Om53, UFRGS Om101, UFRGS Om102, UFRGS Om103, UFRGS Om107, UFRGS Om113, UFRGS Om125, UFRGS Om137, UFRGS Om145
Group 6	UFRGS Om5, UFRGS Om8, UFRGS Om78, UFRGS Om80, UFRGS Om82, UFRGS Om83, UFRGS Om132, UFRGS Om147
Group 7	UFRGS Om59
Group 8	UFRGS Om33, UFRGS Om60, UFRGS Om61, UFRGS Om69, UFRGS Om146
Group 9	UFRGS Om31, UFRGS Om36, UFRGS Om99
Group 10	UFRGS Om100
Group 11	UFRGS Om34, UFRGS Om39, UFRGS Om129, UFRGS Om130
Group 12	UFRGS Om30, UFRGS Om131
Group 13	UFRGS Om133, UFRGS Om134, UFRGS Om139, UFRGS Om144
Group 14	UFRGS Om135
Group 15	UFRGS Om141

greater than or equal to 3 mm colonies within three to four days of growth, which were classified as intermediate growth isolates. The isolates obtained in laboratory were tested in the authentication trials *in vitro* environment for the ability of nodulation and effect on the growth and development of plants. Of the 148 inoculated treatments, 113 were able to nodular the plants of serradella. Isolates that showed higher total fresh weight of plant at the end of the 35 days of experiment were the ones that formed 3 to 5 nodules plant. These are the cases of isolated UFRGS Om9, UFRGS Om62, and UFRGS Om67, from Cachoeirinha (Table 3). These three isolates are highly recommended for studies in the field but when high efficiency is maintained, it will be recommended to the composition of rhizobial inoculants for *Ornithopus micrantus*.

The isolates UFRGS Om25, UFRGS Om26 and UFRGS Om94 formed 12, 3; 12, 3 and 9, 5 nodules per plant, respectively (Table 3). Isolates with the highest number of nodules were less efficient for total fresh mass growth parameter. These results corroborate Souza et al. (2008), which indicate the dry weight of nodules as the best parameter to evaluate nodulation, instead of the number of nodules. Thus, the number of nodules parameter is interesting for the rhizobia authentication, especially in the study of species that form low weight small nodules, but it is not

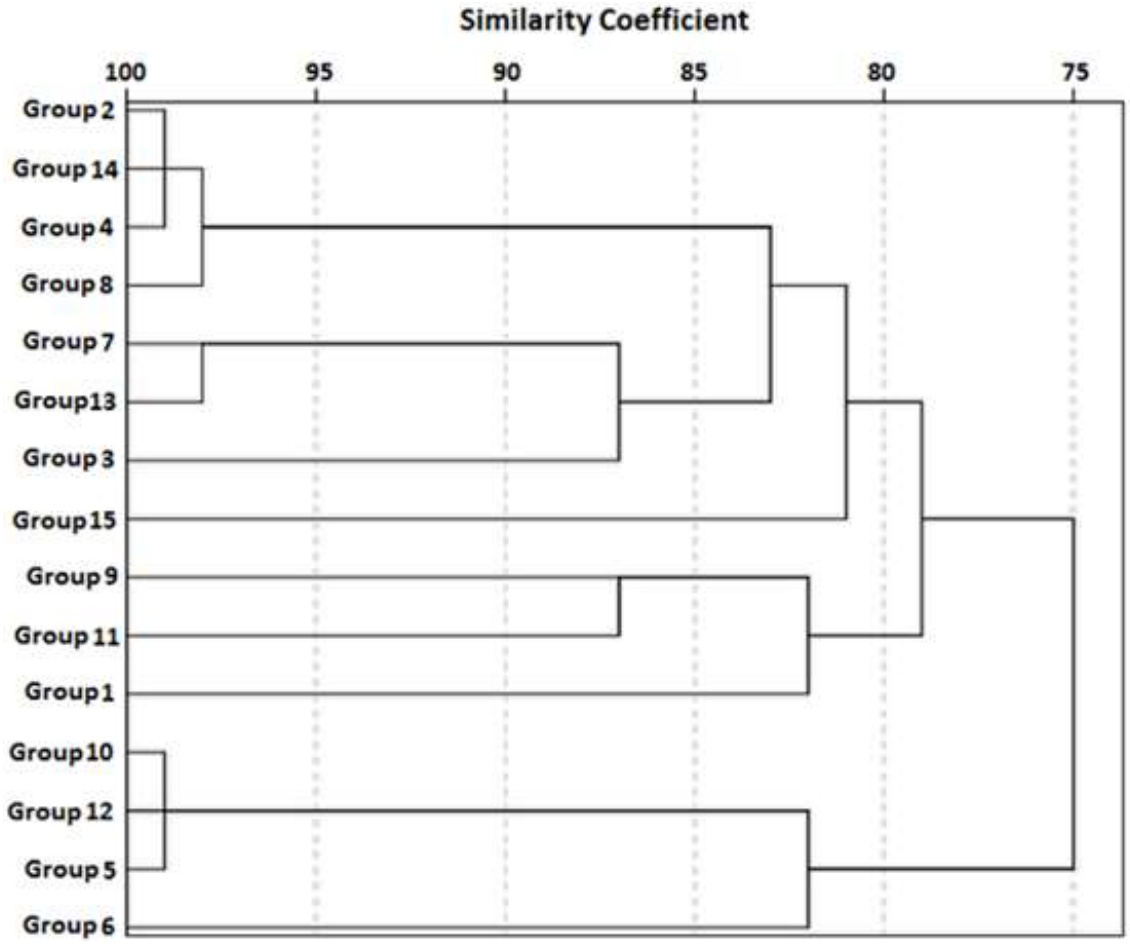

Figure 1. Dendrogram of similarity for the isolates groups, obtained through the Nearest-neighbor method based on morphological characteristics.

suitable to quantify the efficiency of nodulation. As for the total fresh weight parameter, 32 treatments that were superior to the Control treatments wereobtained. 29 of those are from Cachoeirinha, the other three were obtained from collections in the cities Correia Pinto, Passo Fundo and Santa Vitória do Palmar. The best yields were observed in treatments UFRGS Om9 and UFRGS Om62, which showed high total fresh weight of 0,167 and 0,190g, respectively. The best yields treatments (UFRGS Om9 and UFRGS Om62) belonging to group 3 are characterized by slow growth, milky color, diameter smaller than 1 mm, aqueous consistency, opaque and punctiform (Table 1).

The UFRGS Om67 treatment was slightly lower as to the total fresh weight, but overcame those mentioned above, as to the number of leaves, shoot length and root length. Other 16 isolates were able to nodulate plants of serradella, but induced results of total fresh weight, number of leaves, shoot length and root length equal or inferior than the treatment Control - N. It is undesirable that the plants studied are infected by these bacteria, since this interaction does not set up a symbiosis, to the extent that the plant is not benefited by the infection and although it spends photo assimilating metabolites for maintenance of the inefficient nodules. These rhizobia are able to colonize and nodulate serradella plants, but do not contribute to their income, it account for a physiological cost which is not compensated in terms of income. In these cases, it is recommended to exclude these nodulating bacteria from the studies and promote the inoculation of legumes grown with infective rhizobia which are efficient to fix atmospheric N so that, these efficient rhizobia prevail in the sites of infection and promote better yields to plants (Figure 2).The study found positive effect of serradella inoculation with different rhizobia as the isolates UFRGS Om9, UFRGS Om62, and UFRGS Om67 induced significant increases in the yield of serradella plants, especially regarding the

Table 3. Plant fresh mass, relative efficiency index, number of leaves, number of nodules, shoot and root length of serradella (*Ornithopus micrantus*), inoculated with isolates and evaluated after cultivation in vitro by a period of 35 days.

Treatment	Plant fresh mass (mg)	Relative efficiency index (%)	Number of leaves	Number of nodules	Shoot lenght (cm)	Root lenght (cm)
Control N +	33^{d}	100	20.3^{b}	0.0^{e}	7.6^{b}	9.3^{b}
UFRGS Om62	190^{a}	885	22.0^{b}	5.0^{d}	6.6^{c}	7.4^{c}
UFRGS Om9	167^{a}	770	18.0^{c}	3.0^{d}	4.1^{c}	4.9^{d}
UFRGS Om67	130^{b}	585	29.3^{a}	4.3^{d}	11.0^{a}	14.3^{a}
UFRGS Om59	117^{c}	520	26.0^{a}	6.0^{c}	8.4^{a}	9.6^{b}
UFRGS Om4	113^{c}	500	32.0^{a}	6.0^{c}	10.4^{a}	12.8^{a}
UFRGS Om57	110^{c}	485	26.3^{a}	6.0^{c}	9.5^{a}	11.5^{a}
UFRGS Om82	100^{c}	435	25.0^{a}	4.3^{d}	8.5^{a}	12.1^{a}
UFRGS Om148	100^{c}	435	29.3^{a}	6.0^{c}	9.3^{a}	11.9^{a}
UFRGS Om125	93^{c}	400	23.3^{b}	7.3^{c}	9.1^{a}	12.2^{a}
UFRGS Om142	93^{c}	400	22.7^{b}	3.0^{d}	10.0^{a}	11.9^{a}
UFRGS Om74	93^{c}	400	23.3^{b}	3.3^{d}	8.6^{a}	10.3^{b}
UFRGS Om60	90^{c}	385	25.0^{a}	4.3^{d}	8.5^{a}	11.4^{a}
UFRGS Om61	87^{c}	370	23.0^{b}	3.3^{d}	8.3^{b}	9.5^{b}
UFRGS Om58	83^{c}	350	22.3^{b}	5.3^{d}	7.4^{b}	11.2^{b}
UFRGS Om143	80^{c}	335	26.5^{a}	4.5^{d}	9.8^{a}	12.3^{a}
UFRGS Om68	80^{c}	335	24.0^{b}	4.7^{d}	7.6^{b}	6.3^{c}
UFRGS Om80	80^{c}	335	22.3^{b}	5.3^{d}	9.6^{a}	13.4^{a}
UFRGS Om141	77^{c}	320	21.3^{b}	3.3^{d}	6.8^{b}	13.5^{a}
UFRGS Om126	73^{c}	300	21.5^{b}	5.5^{c}	11.5^{a}	10.0^{b}
UFRGS Om146	73^{c}	300	26.3^{a}	4.3^{d}	9.6^{a}	11.6^{a}
UFRGS Om83	73^{c}	300	26.7^{a}	4.7^{d}	9.3^{a}	10.9^{b}
UFRGS Om12	70^{c}	285	33.0^{a}	4.0^{d}	8.2^{b}	10.8^{b}
UFRGS Om13	70^{c}	285	27.7^{a}	5.0^{d}	7.9^{b}	9.0^{b}
UFRGS Om81	70^{c}	285	19.0^{b}	4.0^{d}	8.3^{b}	11.2^{b}
UFRGS Om2	67^{c}	270	25.5^{a}	4.0^{d}	7.8^{b}	9.5^{b}
UFRGS Om25	67^{c}	270	25.7^{a}	12.3^{a}	7.2^{b}	11.6^{a}
UFRGS Om73	67^{c}	270	23.7^{b}	4.3^{d}	7.6^{b}	8.4^{b}
UFRGS Om69	67^{c}	270	22.7^{b}	3.7^{d}	7.2^{b}	7.3^{c}
UFRGS Om65	63^{c}	250	22.3^{b}	3.3^{d}	5.9^{c}	9.3^{b}
UFRGS Om124	63^{c}	250	23.3^{b}	3.3^{d}	8.9^{a}	11.0^{b}
UFRGS Om72	60^{c}	235	21.0^{b}	6.0^{c}	8.7^{a}	10.1^{b}
UFRGS Om5	60^{c}	235	25.3^{a}	3.3^{d}	7.6^{b}	8.9^{b}

Table 3. Contd.

UFRGS Om27	57[d]	220	28.0[a]	8.5[c]	9.0[a]	16.7[a]
UFRGS Om64	57[d]	220	17.0[c]	4.0[d]	5.3[c]	8.0[c]
UFRGS Om76	53[d]	200	21.0[b]	2.6[d]	9.6[a]	7.4[c]
UFRGS Om18	53[d]	200	26.0[a]	4.0[d]	7.4[b]	8.0[c]
UFRGS Om11	53[d]	200	28.7[a]	6.3[c]	7.3[b]	8.0[c]
UFRGS Om134	53[d]	200	16.3[c]	4.3[d]	7.9[b]	6.5[c]
UFRGS Om42	50[d]	185	23.7[b]	3.3[d]	6.8[b]	9.3[b]
UFRGS Om24	50[d]	185	19.7[b]	8.0[c]	4.4[c]	7.1[c]
UFRGS Om123	50[d]	185	17.5[c]	5.0[c]	8.0[b]	10.0[b]
UFRGS Om45	50[d]	185	24.3[b]	5.7[c]	7.2[b]	9.9[b]
UFRGS Om75	50[d]	185	16.5[c]	2.0[e]	11.3[a]	16.5[a]
UFRGS Om20	50[d]	185	23.0[b]	5.3[d]	9.2[a]	9.8[b]
UFRGS Om118	50[d]	185	22.0[b]	2.3[e]	7.1[b]	12.5[a]
UFRGS Om6	50[d]	185	24.7[a]	3.7[d]	7.5[b]	8.1[c]
UFRGS Om131	47[d]	170	16.7[c]	0.0[e]	7.0[b]	7.3[c]
UFRGS Om111	47[d]	170	15.0[c]	0.0[e]	6.1[b]	10.6[b]
UFRGS Om38	47[d]	170	29.0[a]	2.7[d]	6.7[b]	10.2[b]
UFRGS Om138	47[d]	170	21.0[b]	3.0[d]	6.9[b]	6.5[c]
UFRGS Om132	43[d]	150	17.3[c]	0.0[e]	7.4[b]	8.8[b]
UFRGS Om17	43[d]	150	27.3[a]	4.7[d]	7.9[b]	8.8[b]
UFRGS Om117	43[d]	150	19.0[b]	2.3[e]	4.9[c]	6.1[c]
UFRGS Om147	43[d]	150	25.0[a]	3.0[d]	6.1[b]	13.8[a]
UFRGS Om122	43[d]	150	15.5[c]	3.0[d]	6.7[b]	8.8[b]
UFRGS Om116	40[d]	135	17.3[c]	1.0[e]	7.2[b]	10.5[b]
UFRGS Om63	40[d]	135	18.0[c]	3.0[d]	6.3[b]	7.4[c]
UFRGS Om19	40[d]	135	24.0[b]	5.0[d]	5.5[c]	11.6[a]
UFRGS Om140	40[d]	135	14.5[c]	0.0[e]	6.4[b]	10.4[b]
UFRGS Om94	40[d]	135	22.7[b]	12.3[a]	10.1[a]	14.0[a]
UFRGS Om37	40[d]	135	25.0[a]	6.3[c]	5.3[c]	12.1[a]
UFRGS Om56	40[d]	135	19.0[b]	3.3[d]	6.7[b]	7.6[c]
UFRGS Om70	40[d]	135	17.0[c]	2.0[e]	5.6[c]	4.7[d]
UFRGS Om133	37[d]	120	18.3[c]	3.7[d]	7.1[b]	10.8[b]
UFRGS Om119	37[d]	120	19.0[b]	3.0[d]	7.4[b]	9.2[b]
UFRGS Om84	37[d]	120	20.3[b]	4.0[d]	7.2[b]	6.7[c]
UFRGS Om10	37[d]	120	22.3[b]	7.7[c]	5.9[c]	8.8[b]
UFRGS Om14	37[d]	120	21.0[b]	3.5[d]	6.6[b]	7.4[c]

Table 3. Contd.

UFRGS Om71	37[d]	120	17.0[c]	3.0[d]	6.6[b]	5.0[d]
UFRGS Om135	37[d]	120	12.0[c]	2.0[e]	5.7[c]	5.3[d]
UFRGS Om77	37[d]	120	16.0[c]	1.3[e]	6.2[b]	7.2[c]
UFRGS Om79	37[d]	120	22.3[b]	5.0[d]	7.6[b]	7.6[c]
UFRGS Om33	33[d]	100	23.0[b]	3.3[d]	6.3[b]	7.1[c]
UFRGS Om3	33[d]	100	20.3[b]	6.7[c]	4.4[c]	8.0[c]
UFRGS Om1	33[d]	100	19.7[b]	4.7[d]	5.9[c]	7.4[c]
UFRGS Om21	33[d]	100	20.5[b]	3.0[d]	7.2[b]	5.7[d]
UFRGS Om16	33[d]	100	25.0[a]	3.5[d]	7.4[b]	7.6[c]
UFRGS Om40	33[d]	100	18.5[c]	0.0[e]	5.9[c]	6.0[c]
UFRGS Om7	33[d]	100	24.0[b]	4.3[d]	5.5[c]	7.9[c]
UFRGS Om129	33[d]	100	12.3[c]	0.0[e]	7.2[b]	4.6[d]
UFRGS Om78	33[d]	100	13.0[c]	0.7[e]	6.2[b]	4.3[d]
UFRGS Om87	30[d]	85	26.0[a]	3.7[d]	9.3[a]	9.9[b]
UFRGS Om54	30[d]	85	22.3[b]	4.7[d]	6.1[b]	5.2[d]
UFRGS Om110	30[d]	85	18.0[c]	3.7[d]	4.5[c]	9.2[b]
UFRGS Om89	30[d]	85	21.7[b]	4.0[d]	9.4[a]	10.2[b]
UFRGS Om97	30[d]	85	18.0[c]	5.7[c]	7.7[b]	10.5[b]
UFRGS Om26	30[d]	85	19.5[b]	9.5[b]	4.8[c]	10.9[b]
UFRGS Om127	27[d]	70	15.7[c]	1.7[e]	6.4[b]	8.3[b]
UFRGS Om52	27[d]	70	23.0[b]	7.3[c]	7.8[b]	9.3[b]
UFRGS Om22	27[d]	70	14.7[c]	4.0[d]	5.1[c]	5.0[d]
UFRGS Om106	27[d]	70	15.3[c]	5.7[c]	6.6[b]	10.3[b]
UFRGS Om36	27[d]	70	16.3[c]	0.0[e]	3.9[c]	7.6[c]
UFRGS Om137	27[d]	70	8.0[d]	0.0[e]	3.0[d]	3.6[d]
UFRGS Om86	27[d]	70	24.3[b]	3.0[d]	9.6[a]	11.5[a]
UFRGS Om105	27[d]	70	15.3[c]	5.0[d]	5.8[c]	8.8[b]
UFRGS Om23	27[d]	70	19.5[b]	6.7[c]	4.8[c]	5.5[d]
UFRGS Om115	27[d]	70	15.0[c]	0.0[e]	4.8[c]	7.3[c]
UFRGS Om34	27[d]	70	15.7[c]	0.0[e]	4.9[c]	7.8[c]
UFRGS Om41	23[d]	50	17.0[c]	1.3[e]	3.7[c]	3.8[d]
UFRGS Om47	23[d]	50	15.7[c]	2.0[e]	3.4[d]	3.1[d]
UFRGS Om55	23[d]	50	15.3[c]	2.7[d]	5.2[c]	4.4[d]
UFRGS Om90	23[d]	50	19.7[b]	6.7[c]	8.2[b]	10.4[b]
UFRGS Om44	23[d]	50	14.7[c]	0.0[e]	4.1[c]	6.9[c]
UFRGS Om46	23[d]	50	16.7[c]	4.3[d]	4.0[c]	4.8[d]

Table 3. Contd.

UFRGS Om88	23^{d}	50	20.0^{b}	4.3^{d}	8.9^{a}	9.9^{b}
UFRGS Om112	23^{d}	50	13.3^{c}	0.7^{e}	4.1^{c}	5.5^{d}
UFRGS Om113	20^{d}	35	2.5^{d}	0.0^{e}	2.7^{d}	3.3^{d}
UFRGS Om43	20^{d}	35	16.0^{c}	0.0^{e}	3.9^{c}	8.4^{b}
UFRGS Om39	20^{d}	35	15.7^{c}	0.0^{e}	3.7^{c}	5.0^{d}
UFRGS Om103	20^{d}	35	12.0^{c}	0.0^{e}	4.5^{c}	7.8^{c}
UFRGS Om98	20^{d}	35	7.5^{d}	3.0^{d}	4.1^{c}	6.7^{c}
UFRGS Om85	20^{d}	35	15.7^{c}	3.0^{d}	6.5^{c}	6.0^{c}
UFRGS Om50	20^{d}	35	24.3^{a}	3.7^{d}	7.0^{b}	6.7^{c}
UFRGS Om15	20^{d}	35	16.7^{c}	3.3^{d}	4.8^{c}	5.0^{d}
UFRGS Om120	20^{d}	35	16.0^{c}	0.7^{e}	4.9^{c}	7.0^{c}
UFRGS Om99	20^{d}	35	12.0^{c}	0.0^{e}	7.1^{b}	9.3^{b}
UFRGS Om91	20^{d}	35	19.7^{b}	3.7^{d}	9.9^{a}	8.9^{b}
UFRGS Om130	20^{d}	35	6.0^{d}	0.0^{e}	2.0^{d}	4.0^{d}
UFRGS Om29	20^{d}	35	21.7^{b}	5.3^{d}	6.2^{c}	8.1^{c}
UFRGS Om128	17^{d}	20	16.3^{c}	2.0^{e}	7.5^{b}	4.9^{d}
UFRGS Om92	16^{d}	15	21.0^{b}	4.0^{d}	7.1^{b}	10.5^{b}
UFRGS Om93	16^{d}	15	16.3^{c}	3.7^{d}	6.7^{b}	10.0^{b}
UFRGS Om144	16^{d}	15	9.3^{d}	0.0^{e}	2.3^{d}	3.7^{d}
UFRGS Om114	16^{d}	15	14.3^{c}	1.0^{e}	4.2^{c}	2.8^{d}
UFRGS Om95	16^{d}	15	15.7^{c}	6.0^{c}	5.9^{c}	7.3^{c}
UFRGS Om51	16^{d}	15	17.0^{c}	0.0^{e}	5.3^{c}	7.8^{c}
UFRGS Om100	16^{d}	15	13.0^{c}	1.3^{e}	5.9^{c}	10.3^{b}
UFRGS Om28	16^{d}	15	17.3^{c}	7.0^{c}	5.1^{c}	10.3^{b}
UFRGS Om109	16^{d}	15	14.7^{c}	0.7^{e}	7.4^{b}	8.8^{b}
UFRGS Om8	16^{d}	15	16.0^{c}	4.0^{d}	4.8^{c}	5.9^{c}
UFRGS Om108	13^{d}	0	15.3^{c}	1.7^{d}	3.9^{c}	7.1^{c}
UFRGS Om139	13^{d}	0	7.3^{d}	0.0^{e}	3.7^{c}	5.7^{d}
UFRGS Om30	13^{d}	0	22.3^{b}	0.0^{e}	4.6^{c}	8.5.^{b}
UFRGS Om96	13^{d}	0	14.3^{c}	1.7^{e}	5.1^{c}	7.1^{c}
UFRGS Om136	13^{d}	0	12.5^{c}	0.0^{e}	3.0^{d}	2.4^{d}
UFRGS Om101	10^{d}	-15	8.0^{d}	0.0^{e}	2.0^{d}	3.1^{d}
UFRGS Om35	10^{d}	-15	8.0^{d}	0.0^{e}	1.9^{d}	3.4^{d}
UFRGS Om53	10^{d}	-15	8.0^{d}	0.0^{e}	1.9^{d}	3.8^{d}
UFRGS Om48	10^{d}	-15	14.8^{c}	3.6^{d}	4.3^{c}	5.5^{d}
UFRGS Om104	10^{d}	-15	12.7^{c}	0.0^{e}	5.8^{c}	7.5^{c}

Table 3. Contd.

UFRGS Om31	10[d]	-15	8.0[d]	0.0e	2.0[d]	4.2[d]
UFRGS Om107	10[d]	-15	6.3[d]	0.0e	1.7[d]	3.0[d]
UFRGS Om32	10[d]	-15	7.3[d]	0.0e	2.1[d]	4.0[d]
UFRGS Om145	7[d]	-30	8.0[d]	0.0e	1.3[d]	2.7[d]
UFRGS Om121	7[d]	-30	8.0[d]	1.0e	2.8[d]	3.1[d]
UFRGS Om102	7[d]	-30	6.7[d]	0.0e	1.2[d]	2.7[d]
UFRGS Om49	7[d]	-30	18.0[c]	3.3[d]	2.9[d]	4.1[d]
UFRGS Om66	0[d]	-30	11.5[d]	3.0[d]	3.9[c]	7.5[c]
Control N -	13[d]	0	11.0[d]	0.0e	3.7[c]	7.2[c]
CV (%)	61.53	-	17.84	51.59	27.95	28.87

Averages followed by the same letter in the column do not differ among themselves by Scott-Knott test at 5% probability. Control N +: non-inoculated control treatment with the addition of the equivalent to 100 kg of N ha^{-1}; Control N -: non-inoculated control treatment without addition of nitrogen.

increase of root and shoot dry weight.

Evaluation of symbiotic efficiency of rhizobia in serradella plants

The results produced by serradella plants grown in the greenhouse are presented in Table 4. It observed a higher dry weight of shoot (DWS) and root dry weight (RDW) from uninoculated treatment + Nitrogen (Control + N) which is equivalent to the application of 250 kg N ha^{-1}. The inferior yield to the Control + N are, the UFRGS Om57, Om59 and Om148 treatments, which exceeded the Control treatment - N and all other inoculated treatments as DWS and RDW, surpassing the strains SEMIA 905 and SEMIA 929, currently licensed by MAPA (Ministry of Agriculture, Livestock and Food Supply) for the production of inoculants for serradella. Despite the lower total fresh weight of UFRGS Om57, Om59 and Om148 treatments compared to the Control + N, it was observed that these isolates were equivalent to Control + N on the leaf N accumulation, which received N dose equivalent to 250 $kg.ha^{-1}$. This shows the great efficiency of the isolated UFRGS Om57, Om59 and Om148 in biologically fixing atmospheric nitrogen in serradella plants, enabling the elimination of mineral nitrogen fertilization, without prejudice to the total nutrient content of the leaf. In the assessment made at the end of 60 days after plant emergence, the average number of nodes per pot ranged from 0.0 (SEMIA905 and SEMIA929 strains) and 143.0 (UFRGS Om59). The plants of higher dry weight of nodules were inoculated with rhizobia UFRGS Om57, Om59 and Om148, precisely those that also had higher root dry weight (RDW), dry weight of shoot (DWS) and higher total nitrogen content of shoot (N Total). It is inferred that, the most massive nodules rhizobia were the same as an induced greater mass of plants and nitrogen content in the leaves.The treatments UFRGS OM4, Om27 and Om82, was found to have a large number of nodes (NN) and early nodular (EN) in the treatments which the shoot dry weight, root dry weight and N Total were equivalent to Control - N, therefore unsatisfactory. This indicates that many nodules and early nodular recorded did not set atmospheric N and so the physiological costs of these nodules were not converted to increase yield of plants. Thus, in this study we did not observe the connection between the number of nodes and the mass increase of plants or N Total of leaves.

The relative efficiency index (REI %) presented in percentage shows how efficient the symbiont organism is to fix atmospheric N for the legume in association. In percentage terms, the N content obtained in the leaves of each inoculated treatment is compared to the respective values obtained in the leaves of Control + N and Control - N treatments, to estimate the potential of each isolate to fix atmospheric N under axenic conditions. Isolates UFRGS Om57; Om59 and

Figure 2. Serradella inoculated with effective rhizobia, 35 days after emergence (left) and serradella not inoculated, 35 days after emergence (right).

Table 4. Dry weight of shoot (DWS), root dry weight (RDW), nodules dry mass (NDM), total nitrogen of shoots (N Total), Relative Efficiency Index (REI), number of nodules (NN) and number of early nodules (EL) of serradela (*Ornithopus micranthus*), grown in the greenhouse. Average of four replications, with two plants per pot.

Treatment	DWS (mg)	RDW (mg)	NDM (mg)	N Total(mg)	REI (%)	NN*	EL*
Control + N	1997^{a}	767^{a}	0^{c}	23.9^{a}	100.0	0^{b}	0^{b}
UFRGS Om148	1237^{b}	234^{b}	45^{a}	27.7^{a}	117.3	127^{a}	29^{a}
UFRGS Om59	1089^{b}	303^{b}	34^{a}	26.7^{a}	112.9	143^{a}	43^{a}
UFRGS Om57	967^{b}	188^{b}	32^{a}	24.9^{a}	104.5	132^{a}	39^{a}
SEMIA 929	654^{c}	125^{c}	0^{c}	6.0^{c}	19.3	0^{b}	0^{b}
UFRGS Om67	609^{c}	158^{c}	23^{b}	14.7^{b}	58.6	86^{a}	32^{a}
UFRGS Om62	570^{c}	190^{b}	22^{b}	13.7^{b}	53.7	115^{a}	44^{a}
UFRGS Om82	338^{c}	133^{c}	14^{c}	7.8^{c}	27.1	79^{a}	28^{a}
UFRGS Om27	240^{c}	95^{c}	14^{c}	3.5^{c}	7.7	116^{a}	25^{a}
UFRGS Om4	235^{c}	81^{c}	6^{c}	2.7^{c}	4.2	71^{a}	43^{a}
SEMIA 905	196^{c}	196^{b}	0^{c}	1.6^{c}	-0.6	0^{b}	0^{b}
Control - N	205^{c}	97^{c}	0^{c}	1.8^{c}	0.00	0^{b}	0^{b}
CV (%)	61.01	36.07	62.13	56.96	-	44.73	28.6

Control + N: treatment not inoculated, fertilized with nitrogen dose (N) equivalent to 250kg N.ha^{-1}; Control - N: treatment not inoculated, without N fertilization

Om148, (RER%) indexes exceeding 100%: 104.5%; 112.9 % and 117.3 %, respectively, were observed. With these results, it is shown that these symbiotic associations were efficient in the increase of total N content in the leaves, being superior to the strains of SEMIA collection and thus promising for future studies in order to be recommended for the production of commercial inoculants to serradella.

SEMIA905 and SEMIA929 strains did not form nodules in serradella (*Ornithopus micranthus*) nor induced growth in serradella plants studied. Thus, based on the present results, it is inferred that in serradella plants (*Ornithopus micranthus*) better yields are obtained with the placement of the isolated UFRGS Om57, Om59 and Om148.

DISCUSSION

Rhizobacteria that promote plant growth are inserted into pasture production systems for, feeding cattle that produce meat or milk. As they present mechanisms that promote plant growth, they may be recommended for the composition of commercial inoculants for specific crops. In the case of the use of rhizobacteria in pastures, these plants yield increment mechanisms should somehow be able to anticipate the supply of pastures, lengthen the crop production cycle, or increase the supply of pastures to animals the same time they keep or reduce the need for use of mineral fertilizers.

With this study we found positive effect of inoculation of different rhizobia on the yield of serradella (*Ornithopus micranthus*). Isolated UFRGS Om57; Om59 and Om148 induced significant increases in the yield of serradella plants, especially regarding the increase of the dry weight of shoot and root dry weight. For the isolated UFRGS Om57, UFRGS Om59 and UFRGS Om148, the relative efficiency index (RER %) for accumulation of total nitrogen (N) in shoots was greater than 100%. These results demonstrate the high efficiency of these isolated (Table 3) which were higher than those found in the literature, as the study of symbiosis between rhizobia grown forage legumes (Scheffer-Basso et al., 2001; Frizzo, 2007) and (Scheffer-Basso et al., 2001). Fontoura et al. (2011) obtained similar results with the inoculation of rhizobia in *Lotus glaber*.

The use of these high performance diazotrophic bacteria significantly increase in N supply to the soil, eliminating the mineral nitrogen fertilizer without damage to the crop yield and to the N supply to the soil. Additionally, some rhizobia are known to produce phyto stimulating substances, such as hormones from the auxin group (Anjum et al., 2011; Spaepen and Vanderleyden, 2011), cytokinins (Senthilkumar et al., 2009) and gibberellins (Erum and Bano, 2008), which are associated with an increase in the yield of legumes and non-legumes. Thus, investment in the rhizobia inoculants that optimize the quality and quantity of pastures offered to cattle that produce milk or meat, may increase the productive performance of the animals.

Moreover, due to the specificity between the host plant and the rhizobium, for both rhizobia fasteners of atmospheric N, and for rhizobia producers of plant stimulating substances, the study of the interaction and the effect of inoculation of these organisms for the yield of plants are required. In this study it was observed that the specificity within the *Ornithopus* gender, as strains recommended for the composition of commercial inoculants for use in pastures composed of *Ornithopus sativus* showed no nodulation and had poor performance on the biological nitrogen fixation and growth promotion of the *Ornithopus micranthus* species (Table 3). Based on these results, we highlight the importance of studies on specificity between bacteria and plant; there is no affinity between SEMIA905 and SEMIA929 strains and serradella *Ornithopus micranthus*.

As for savings promoted with the use of effective rhizobia, we can assume a scenario based on economic data seen in the first quarter of the year 2016 in the state of Rio Grande do Sul, Brazil: considering the price of the US dollar (US$) in R$ 3.487 in March 2016 which state that, the price of urea in the Brazilian domestic market was approximately US$ 200.00 ton^{-1}. Based on this study after 60 days, the inoculation of serradella with fixing bacteria of N (UFRGS Om57; Om59 and Om148) would supply the equivalent of 250 kg $N.ha^{-1}$ (Table 3), representing a saving of U$ 111.11 ha^{-1} in this scenario. Knowing that the average productivity of dairy herd of Rio Grande do Sul state lies at 7.9 liters of milk / cow / day (IBGE, 2013); that in March 2016, the average price of a liter of milk in the state of Rio Grande do Sul was $ 0.31 (CEPEA/ESALQ, 2016); and assuming a stocking of 7 Units Animals/ha, at the end of 60 days, milk production in 1 ha would have a gross value of US$ 1,028.58. The value of N fixed biologically would be equivalent to 10.8% of the gross value of the production obtained by the exploration of the dairy cattle in 1 ha, for 60 days. This economy would surely bring greater economic sustainability for dairy cattle farm in the state of Rio Grande do Sul, favoring the permanence of small milk producers in the dairy business.

Conclusions

We obtained and described 148 isolate, of which 113 were capable of forming nodules in association with *Ornithopus micranthus*. The isolates UFRGS Om57, UFRGS Om59 and UFRGs Om148 were equivalent to Control + N on the leaf N accumulation, which received N dose equivalent to 250 $kg.ha^{-1}$ in greenhouse. This shows the great efficiency of the isolated UFRGS Om57, UFRGS Om59 and UFRGS Om148 in biologically fixing atmospheric nitrogen in serradella plants, enabling the elimination of mineral nitrogen fertilization, without prejudice to the total nutrient content of the leaf.

The isolates that had better performance were characterized with slow growth in culture medium and punctiform colonies with diameter smaller than one millimeter. These characteristics are typical example of genus *Bradyrhizobium*.

Conflict of Interests

The authors have not declared any conflict of interests.

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REFERENCES

Anjum MA, ZAHIR ZA, ARSHAD M, ASHRAF M (2011) Isolation and screening of rhizobia for auxin biosynthesis and growth promotion of bean (*Vigna radiate* L.) seedlings under axenic conditions. Soil Environ. 30:18-26.

Beck DP, Materon LA, Afandi, F (1993). Practical rhizobium-legume technology manual. International Center for Agricultural Research in Dry Areas (ICARDA): Aleppo. P 389.

Brockwell J, Hely FW, Neal-Smith CA (1966). Some symbiotic characteristics of rhizobia responsible for spontaneous, effective field nodulation of *Lotus hispidus*. Aust. J. Exp. Agric. Anim. Husb. 6:365-370.

Buck JD, Cleverdon RC (1960). The spread plate as a method for the enumeration of marine bacteria. Limnol. Oceanogr. 5:78-80

CEPEA: Centro de Estudos Avançados em Economia Aplicada – ESALQ/USP (2016). Boletim do Leite. n. 252. Piracicaba, P 8. Available at: http://www.cepea.esalq.usp.br/leite/boletim/252.pdf

Conn HJ, Jennison W, Weeks, OB (1957). Routine tests for the identification of bacteria. In: Manual of microbiological methods. Conn, HJ, Pelezar MJ. Massachusets: McGraw-Hill Book Company, pp. 140-168.

Dartora V, Kramer A, Dalastra DJ, Neto FM, Bruschi N, Fruscalso V, Leitzke VW, Gasparin W (2012). Curso bovinos de leite. Emater/RS-ASCAR: Porto Alegre. P 89.

Erum E, Bano A (2008). Variation in phytohormone production in *Rhizobium* strains at diferent altitudes of north areas of Pakistan. Int. J. Agric. Biol. 10:536-540.

Fernandes A, Reis A (2001). Serradela *(Ornithopus sativus)* - Ficha técnica 96. Braga: Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, p.2. Available online: http://www.drapn.min-agricultura.pt/drapn/conteudos/FICHAS_DRAEDM/Ficha_tecnica_096_2001.pdf

Ferreira DF (2000). Sistemas de análise estatística para dados balanceados. UFLA/DEX/SISVAR: Lavras. P 145.

Fontoura RA, Frizzo MLS, Sá ELS, Osório Filho BD, Tonon BC, Binz A, Silva MC, Camargo FAO, Selbach PA (2011). Rizóbios nativos do Rio Grande do Sul simbioticamente eficientes em *Lotus glaber*. Rev. Ci. Rural 41:440-446.

Freixial RMC, Barros JFC (2012) Pastagens. Évora: Universidade de Évora, Escola de Ciências e Tecnologia, Departamento de Fitotecnia. p.38. Available online: https://dspace.uevora.pt/rdpc/bitstream/10174/5107/1/Sebenta%20Pastagens.pdf

Frizzo MLS (2007). Seleção e Caracterização de rizóbios nativos, de solos do Rio Grande do Sul, para *Lotus corniculatus* L. e *Lotus uliginosus*Schkuhr. Masters Dissertation, Programa de Pós-Graduação em Ciência do Solo, Universidade Federal do Rio Grande do Sul, Porto Alegre. P 68.

IBGE (2013). Produção de pecuária municipal. Rio de Janeiro: IBGE, Diretoria de Pesquisas, Coordenação de Agropecuária. P 100.

Jandrey DB (2008). Doses de nitrogênio em cobertura no arroz irrigado em sucessão a espécies de inverno, Masters Dissertation, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre. P 65.

Menezes VG, Mariot CHP, Lopes MCP, Silva PRF, Teichmann LL (2001). Semeadura direta de genótipos de arroz irrigado em sucessão a espécies de cobertura de inverno. Pesq. Agropec. Bras. 36:1107-1115.

Miles AA, Misra SS (1938). The estimation of the bacterial power of blood. J. Hyg., 1938, 38: 732-749. Moura RA, Wada CS, Purchio A, Almeida TV (1987). Técnicas de laboratório. 3rd ed. Rio de Janeiro. P 511.

Sarruge JR (1975). Soluções nutritivas. Summa Phitopathol. 1:231-234.

Scheffer-Basso SM, Jacques, AVA, Dall´Agnol M, Riboldi J, Castro SMJC (2001). Disponibilidade e valor ntritivo de forragem de leguminosas nativas (*Adesmia* DC.) e exóticas (*Lotus* L.). Rev. Bras. Zootecn. 30:975-982.

Senthilkumar M, Madhaiyan M, Sundaram SP, Kannaiyan S (2009). Intercellular colonization and growth promoting effects of *Methylobacterium* sp. with plant-growth regulators on rice (*Oryza sativa* L. CvCO-43). Microbiol. Res. 164:92-104.

Spaepen S, Vanderleyden J (2011). Auxin and plant-microbe interactions. Cold Spring Harb. Perspect. Biol. 3(4):a001438.

Souza RA, Hungria M, Franchini JC, Maciel CD, Campo RJ, Zaia DAM (2008). Conjunto mínimo de parâmetros para avaliação da microbiota do solo e da fixação biológica de nitrogênio pela soja. Pesq. Agropec. Bras. 43:83-91.

Tedesco JM, Gianelo C, Bissani CA, Bohnen H, Volkweiss SJ (1995). Análises de solo, plantas e outros materiais. Universidade Federal do Rio Grande do Sul: Porto Alegre. P 174.

Vincent JM (1970). Manual for the practical study of root nodule bacteria. Blackwell Scientific: Oxford. P 164.

Yanni YG, Dazzo FB (2010) Enhancement of rice production using endophytic strains of *Rhizobium leguminosarum* bv *trifolii* in extensive field inoculation trials within the Egypt Nile delta. Plant Soil. 336:129-42.

Yanni YG, Dazzo FB, Zidan MI (2011) Beneficial endophytic rhizobia as biofertilizer inoculants for rice and the spatial ecology of this bacteria-plant association. In: Maheshwari DK (eds) Bacteria in Agrobiology: Crop Ecossystems. Springer Berlin Heidelberg. pp. 265-294

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Optimization of parameters to increase the xylose reductase production from *Candida tropicalis* strain LY15 using corn cob as hemicellulose waste substrates

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Xylose reductase (XR) is a key enzyme in order to obtain xylitol from xylose. It has vast applications in biotechnology including xylitol production. The present study aimed to estimate and characterize XR from *Candida tropicalis* strain LY15. *C. tropicalis* strain LY15 showed xylose utilization ability and xylose reductase activity after 48 h of incubation at pH 6.5, incubation temperature 28°C and rotation speed 140 rpm. It was specific to nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) with an activity of 32.13 IU/ml. Response surface methodology (RSM) was taken into account in order to determine the effect of four main factors, that is, inoculum (1%), hemicellulose waste substrates (HWs) (2%), incubation period (60 h), and RPM (140) on enzyme production. The maximum XR enzyme activity on corn cob substrates (62.80 IU/ml) was found. The xylitol yield (12.08 g/L) attained corn cob media after 60 h of fermentation. Three dimensional response and interaction plot of the quadratic model showed interdependent interaction between the effective variables. Analysis of variance (ANOVA) predicts R^2 value close to 1 which makes the result highly significant ($p \leq 0.0001$). These values were higher when compared with the traditional fermentation processes.

Key words: *Candida tropicalis*, corn cob, xylitol, response surface methodology (RSM), xylose reductase.

INTRODUCTION

Yeasts have played an important role in industrial development for thousands of years. Yeast is a group of fungi predominantly containing unicellular form. Though, yeast of different genera *Kloeckera*, *Hansensiaspora*, *Candida,* and *Pichia* are involved, but in most cases, *Saccharomyces* species dominate the final stage of the fermentation than any other yeast species (Heard and Fleet, 1985). Many types of yeasts are used in food, baking industries and fermentation for xylitol production. In general, fungi are thought to degrade oligosaccharides, initially obtained from complex polymers (Pérez et al., 2002). Xylose reductase (XR) is

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vital enzyme effective during the preliminary step in the metabolism of xylose (Moysés et al., 2016). The enzyme catalyzed the xylose, which can be further converted into xylulose. Xylitol has been considered to trigger the expression of xylanolytic enzymes (Margolles-Clark et al., 1997; Usvalampi, 2013). In fact, xylitol is a naturally occurring sugar alcohol. The calories of xylitol are less when compared with sucrose based upon on mass and has similar sweetness, which permit its applications in food industries with anticarcinogenic activities, or in medicines (Saha et al., 1997). Xylans are converted into xylitol, a potential substitute of sugar for diabetic patients (Barthikannan et al., 2016).

The metabolism of Xylitol is independent of insulin that can be applied in the food and pharmaceutical areas. The catalytic hydrogenation of D-xylose results into xylitol (Paidimuddala and Gummadi, 2014). Unlike bacteria and fungi, yeasts have been widely used for the production of xylitol. Bacteria do not metabolize xylitol in the mouth and thus, prevent the tooth decay. Furthermore, xylitol is a potential sugar substitute for diabetic patients.

The xylose can be reduced to xylitol by reusing the yeast cells (Tamburini et al., 2015). Yeast reduces D-xylose to xylitol using key XR in the presence of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and metabolizes the extracellular xylose to xylitol in a step that consumes NADPH (Parajo et al., 1998). Xylitol is a natural metabolic intermediate product for xylose consuming microorganisms that makes the process cost-effective and suitable for industrial purposes due to easy cultivation of microbes at any parameters (Silva et al., 1998). Xylitol production through enzymatic approach obtains a substantial enhancement in its productivity. *Candida boidinii* (Vandeska et al., 1995), *Candida parapsilosis* (Oh et al., 1998), *Candida guilliermindii* (Zagustina et al., 2001; Rodrigues et al., 2003), *Candida peltata* (Saha and Bothast, 1999) and *Candida tropicalis* (Kim et al., 2002; Lopez et al., 2004) are well-known for potential xylitol producer. Medium containing 35% xylose mother liquor hydrogenates sugars favours the growth of *Candida maltosa* ATCC28140, that is, it converts xylose to xylitol (Lin et al., 2010). Xylitol can also be obtained by microbial transformation mechanism (Izumori and Tuzaki, 1998; Fredlund et al., 2002).

According to the report, *Saccharomyces cerevisiae* (recombinant) with XYL1 gene encoding XR from *Pichia stipites* converts produces xylitol using glucose (Hallborn et al., 2001). Yeast isolates have lots of advantage as biocatalysts (Pscheidt et al., 2008). Current literatures include several reports of production from various substrates, but very few studies discuss exploitation of novel yeast isolates producing xylitol (Altamirano et al., 2000). Optimization is important for enriched production of enzyme rely on medium components like carbon source, nitrogen sources, pH, temperature, agitation, HWs and incubation time. The optimization of the fermentation medium by one factor at a time (OFAT) is not only a time intense technique but also may lead to imprecise results and conclusions. This standard way of optimization is unable to detect the interaction amongst two factors responsible for upgraded enzyme production (Ayadi et al., 2016). Response surface methodology (RSM) a statistical approach helps to maximize the enzyme yield by designing limited tests for several parameters (Khusro et al., 2016).

This investigation was focused to determine the influence of initial substrate concentration in *C. tropicalis* strain LY15 fermentations using different Hemicellulose Waste substrates as carbon source. A statistical model was used to determine the optimum parameters to produce maximum XR enzyme using *C. tropicalis* strain LY15 using corn cob, which is a low-cost cost substrate.

MATERIALS AND METHODS

Microorganism

C. tropicalis strain LY15 was isolated and identified from *Persea americana* (Butter fruit) in previous studies (Barathikannan et al., 2016). For the fermentation experiments, the yeast *C. tropicalis* strain LY15 (KJ734199) was incubated at 4°C in yeast extract peptone dextrose (YEPD) slants. The production medium contained Yeast Extract Dextrose Agar (Yeast Extract 1%, Dextrose 2%, Agar 2%, pH 6.5; Himedia, Mumbai, India; pH: 7.0±0.2 and Chloramphenicol: 100 mg/L) medium and maintained at 30°C for 24 to 48 h. The isolates were cultured on YED agar to get pure cultures and they were kept as slants supplemented with chloramphenicol for further studies.

Xylose assimilation tests

Xylose assimilation tests were carried out in the medium (yeast extract 20 g/L, xylose 20 g/L, agar 25 g/L). These experiments were performed on both solid and liquid media. The cells were pre-cultured in YED (dextrose, 20 g/L) medium at 28°C and 140 rpm). The pre-cultures were diluted up to 10^{-5} times and inoculum of each culture was spread on the YPX (xylose, 20 g/L) medium. The total colonies appeared on the plate was counted after the incubation.

Analytical method

Xylose and xylitol concentrations were determined using dual-detection HPLC analytical method using Agilent 1100 series HPLC (Agilent 1100; Hewlett-Packard, Waldbronn, Germany) using an anion exchange column (HPX-87H) packed with sulfonated polystyrene-divinyl benzene. Acetonitrile and water (80:20) were used as the mobile phase followed by Barthikannan et al (2016). Xylose and xylitol were used as the standards for the hydrolysates in the bioconversion mechanism.

OFAT for XR

XR production from potential yeast culture was optimized for the following parameters: pH (5-7), Temperature (25 to 35°C), agitation speed (120 to 160), incubation time (12 to 96 h), carbon source

(xylose, glucose, maltose, dextrose) and nitrogen source (peptone, yeast extract, ammonium sulphate, ammonium chloride).

Preparation of cell-free extract

Fermentation was performed at 28°C. After cooling, the culture was inoculated into sterilized medium and cultivated on orbital incubator at 140 rpm and 30°C. After the growth of yeast cells in YEPX (yeast extract peptone xylose) medium, the content was centrifuged at 6,000 rpm at 20°C and cells were washed with autoclaved distilled water. Cell disruption was performed in a homogenizer for 15 min and the supernatant was used after centrifugation at 12,000 rpm.

XR assay

The enzyme activity was calculated spectrophotometrically by UV-Visible (ELICO Double Beam SL-210) spectrophotometer at 340 nm. The final mixture contained (in 1 ml) 600 µl of 250 mM potassium phosphate buffer (pH 7.0), l00 µl of 100 mM mercaptoethanol, 50 µl of 0.5 M D-Xylose, 100 µl of distilled water, 50 µl of 3.4 mM NADPH and the mixture was kept for 1 min. Hundred microliters of enzyme solution were added in order to initiate the reaction. One unit of enzyme activity represents the quantity of enzyme that oxidizes one micromole NAD (P)H in 1 min. The enzyme activity (IU/ml) was calculated for the respective isolates.

Preparation and pretreatment of the agricultural substrates

The three agricultural substrates used in this study were paddy straw, sugarcane bagasse, and corn cob. The substrate particles were sun dried for 48 h, then stored at room temperature in plastic bags for further use. The substrate was ground and sieved for future experiments. The pretreatment experiment was performed in 500-ml conical flasks. Briefly, 10 g of each agricultural substrate was soaked into 2% sodium hydroxide at a solid-liquid proportion of 1:3 and mixed with (0.5% w/w) H_2SO_4 + 1.5% (w/w) H_3PO_4 and pretreatment was carried out at 130°C for 60 min. XR assay was performed as described earlier.

Optimization of different variables for xylose reductase production using three hemicellulose waste substrates by response surface methodology

Box-Behnken design (BBD) was selected to optimize selective independent parameters, viz., inoculum, HWs, time periods and RPM (agitation speed) to maximize XR production or response by keeping pH and temperature constant. The total combinations are $2^k + 2k + n$, where 'k' represents parameters and 'n' corresponds to repetition of runs at the central point.

The experimental design consisted of 29 runs of four variables (A, B, C, D) at three levels (-1, 0, +1) in order to optimize the medium components. The coded values -1 and +1 indicate low and high level of the variables studied based on our previous experiments, respectively. The experimental plan of independent variables is represented in Table 4. The average enzyme activity obtained was considered as (Y). The significance level was validated by *F* test. The desirability was kept at maximum (Table 3).

Experimental design validation

The analysis was validated for enzyme production in shaking flask conditions using optimized parameters of BBD to confirm the experimental value and predicted value of XR production. The experiments were performed in triplicate and enzyme activity was estimated according to the protocol described earlier (Khusro et al., 2016).

Statistical analysis and software

All the experiments were performed in triplicate and data presented are mean ± standard deviation (SD). The independent variables of design were optimized and analyzed using Design Expert Version 7.0.0 (Stat-Ease Inc., Minneapolis, Minnesota, USA) statistical software.

RESULTS

Xylose-assimilating yeasts

C. tropicalis strain LY15 revealed more colony growth on solid medium using xylose. Estimation of intra and extracellular enzymes depicted that the isolates, which showed good growth in xylose supplemented medium consumed xylose in a faster manner. *C. tropicalis* strain LY15 showed maximum consumption of xylose based on the peak and retention time by HPLC.

Effect of different parameters in fermentation condition for xylitol production based on XR assay

In yeast, xylose assimilation is catalyzed by xylitol dehydrogenase and XR. Based on OFAT, different parameters were used for the enzyme production and optimized further at a range of pH, temperature, agitation speed and incubation time. Various carbon and nitrogen sources were also applied to estimate optimal enzyme activity. The isolates *C. tropicalis* strain LY15 was showing maximum enzyme production at pH 6.5, temperature 28°C, agitation speed of 140 rpm for 48 h of incubation. Xylose and dextrose showed similar rate of enzyme production. On the other hand, peptone and yeast extract were potential nitrogen source for XR production. *C. tropicalis* strain LY15 showed enhanced production of XR activity (32.13 IU/ml) among isolates (Figure 1).

Effect of corn cob on production of xylitol

In this present study, the corn cob is better xylitol producer substrates when compared with other HWs (data not shown). The optimized and acid-alkali treated hydrolysate medium constituting (g/L): yeast extract 10, peptone 20, HWs 20 g is a xylitol producing medium. The findings showed that the production of 12.08 g/L of xylitol and XR activity (62.80 IU/ml) in 60 h under optimized parameters.

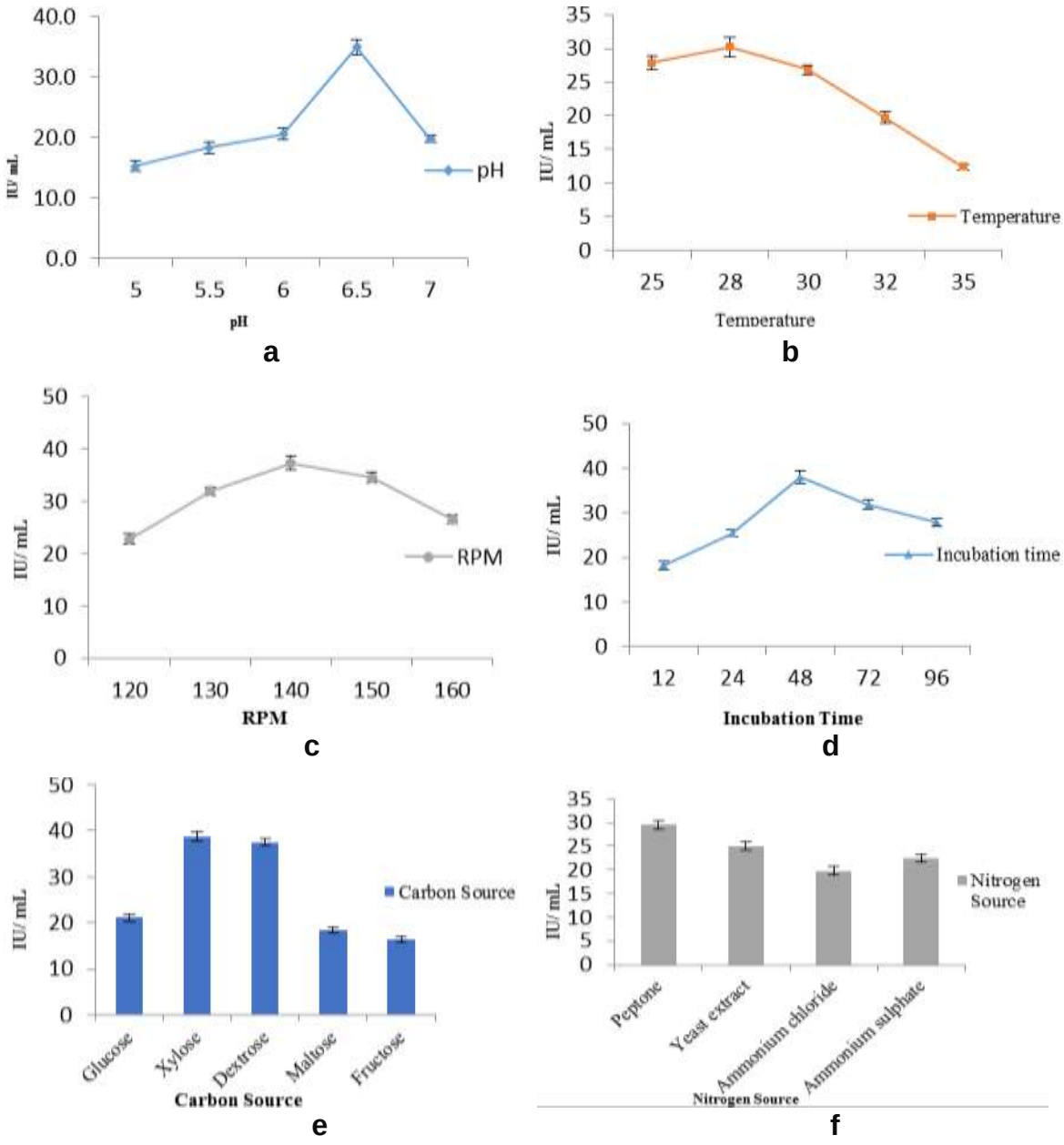

Figure 1. Optimization condition for xylose reductase from *Candida tropicalis* strain LY15. (a) pH, (b) Temperature, (c) RPM, (d) Incubation time, (e) Carbon Source, (f) Nitrogen Source. Each value represents the mean ± SD of triplicate experiments

Three different hemicellulose waste substrates using production of xylitol by RSM

The independent variables such as inoculum (A), HWs concentrations (B), time period (C) and RPM (D) were identified as significant parameters based on the preliminary investigation (Table 1). These variables were further optimized by RSM using Box-Behnken design. Box-Behnken design, consisting of 29 experiments with 3 levels of 4 variables in coded, experimental and predicted values of XR activity is shown in Tables 1 and 3 and Figure 2.

The production of intra enzyme was predicted by the following model:

Y (IU/ml) = 61.64+5.62A+8.21B+9.78C+0.11D-1.22AB-6.35AC-6.55AD 0.58BC+4.98BD-0.95CD-10.68A2-21.65B2-17.45C2-6.05D2

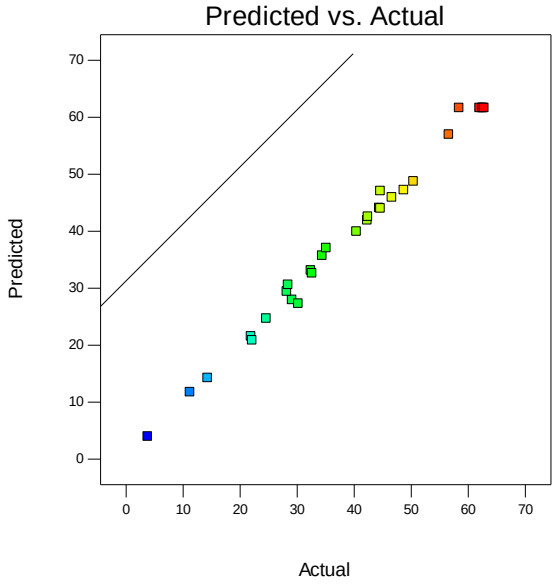

Figure 2. Actual values vs. predicted values for xylose reductase activity residing close to the diagonal line.

where the quadric model represents inoculum (A), HWs (B), time period (C) and RPM (D).

The statistical importance of the second-order model equation was determined by F test. ANOVA for quadric model is given in Table 2. Model terms having *p* value <0.05 were considered significant. The model F value of 120.28 represents significant model. There is only 0.01% chance that a large "Model F value" could happen due to noise. Values of "Prob>F"<0.05 indicate significant model. Here, A, C, A2, B2, and C2 are significant terms (Table 2). The multiple correlation coefficients (R2) denote positive correlation between experimental and predicted values and indicate that model is accurate with better response. A low CV (3.05%) corresponds to reliable and precise experiment. The "Predicted R^2" of 0.9074 agrees with the "Adj R^2" of 0.9853. "Adeq Precision" ratio of 29.785 shows an adequate signal and the model can be applied to navigate the design space.

3D plot (Figure 2) showed interaction between two independent parameters. The response surface was plotted by estimating the enzyme activity against any two factors. The enzyme production varied significantly upon changing the levels of independent variables. The maximum enzyme production was observed with respect to the central values of these independent variables. Maximum enzyme production was obtained when the variables were at their middle to high level.

Validation of model was done by conducting experiments in triplicate using predicted optimized parameters by RSM. Highest XR activity was observed with experiment number 3 using inoculum (1%), HWs (2%), time period (60 h) and RPM (140). The maximum XR production by isolates was 62.80 IU/ml which was found positively correlated with predicted value, that is, 61.64 IU/ml (Figure 3).

DISCUSSION

Yeasts are known as the suitable producers of xylitol and have been broadly studied among xylose-utilizing microorganisms (Barathikannan et al., 2016). Winkelhausen and Kuzmanova (1998) summarized 22 yeasts isolates selected for the production of xylitol. Most of them belong to the genus *Candida*. Rangaswamy and Agblevor (2002) screened bacteria for xylitol production, but xylitol production was found to be very less.

Xylose uptake was reduced with the unavailability of co-substrate. Xylose transport is also affected by the higher concentration of substrate. These disadvantages barred its major role in the production of xylitol at large-scale in spite of the xylitol production approaching the maximum range (Barathikannan et al., 2016). According to Guo et al., (2006), *C. trophicali* and *C. maltosa* APP were potential xylitol producers and showed different modes of xylose assimilation. *C. maltose* Xu316 showed a higher xylose assimilation rate while *C. guiiliermondii* Xu280 had a greater xylitol production and produced less amount of by-product. Our present investigation favours the finding of Altaminaro et al. (2000) who demonstrated

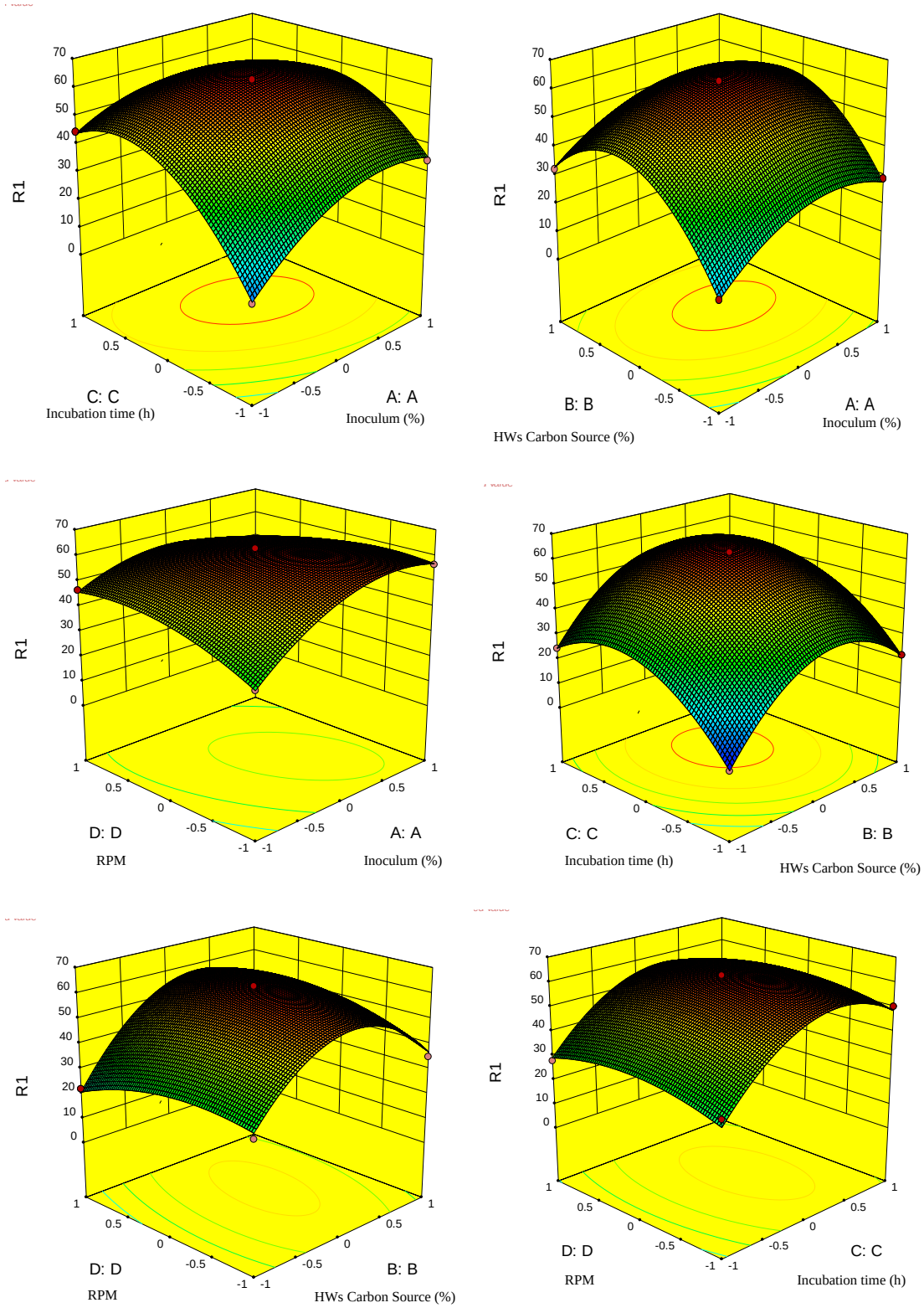

Figure 3. Response surface plot showing interaction between four variables for xylose reductase enzyme activity. (a) Interaction between incubation time and inoculation, (b) Interaction between HWs carbon source and inoculation, (c) Interaction between RPM and inoculation, (d) Interaction between incubation time and HWs carbon source, (e) interaction between RPM and HWs carbon source, and (f) Interaction between RPM and incubation time.

that *C. tropicalis* isolates were found to be a potential producer of xylitol. The rate of xylitol production of our isolates varies from the previous studies, who observed 0.69 g xylitol per gram of xylose. It might be due to the potential of xylitol production by isolates that depends on the compound regulation of the catabolism of xylose even in the same species. *C. tropicalis* strain LY15 had been extensively studied as a XR producer based upon the xylanase, xylose assimilation test and XR, taking part in xylan to xylitol bioconversion mechanism (Barathikannan et al., 2016). XR and xylitol dehydrogenase activity are linked to NADPH and NAD, respectively (Aguiar et al., 2002). The present study shows that there was slight increment in the enzyme production at optimized parameters of different factors. pH is one of the important parameters that changes final product yield. The production of xylitol by *Debaryomyces hansenii* NRRL Y-7426 was optimum between pH 4.5 and 5.5 (Converti et al., 2001; Dominguez et al., 1997). In our study *C. tropicalis* strain LY15 showed enhanced enzyme production at pH 6.5. As *C. tropicalis* strain LY15 showed maximum XR assay (32.13 IU/ml); hence, it is potent yeast for xylitol production, and could be used in industrial process and further isolates improvement process. OFAT optimization method is not a proper method to understand the interaction among the factors which affect the optimization. The exploitation of microorganisms is a cost-effective process for obtaining biotechnologically important products, because of the consumption of renewable sources.

Rafiqul and Sakinah (2015) reported that *C. tropicalis* using the substrates of Meranti wood sawdust hemicellulosic hydrolysate (MWSHH)-based medium produced XR, activity is 11.16 U/m in 24 h (Table 4). In our study, *C. tropicalis* strain LY15 was cultivated using corn cob hydrolysate, showing maximum XR assay 62.80 IU/ml (Xylitol 12.08 g/L) within 60 h. Several findings mentioned the uses of agricultural wastes for xylitol production in the presence of XR (Misra et al., 2013). Different agricultural wastes like, corn fiber, corn stover, rice straw, wheat straws, and sugarcane bagasse, comprise hemicellulose (20 to 40%). The toxic compounds produced during the pretreatment process inhibit the microorganisms (Alvira et al., 2016). Thus, the bioconversion process requires the removal of inhibitors. Many detoxification methods have been used for improving the growth microorganisms (Mussatto and Roberto, 2004). Methods such as over liming (ion exchange resins or activated charcoal adsorption) were found to act as detoxification (Altamirano et al., 2000). In the previous studies, various measures had been taken for the optimization of operation bioprocess parameters. RSM is a potential substitute of OFAT tool which is not only used to optimize the medium components but also helps to understand the combined interaction of all the independent variables of a fermentation process.

According to the present study, the significant factor involved in the maximum production of enzyme from isolates was further optimized by RSM using BBD. BBD is an optimization method for small number of variables that estimates best fit parameters of the quadratic models. It detects not only the lack of fit of the model but also construct a sequential design for response surface methodology. The optimization of temperature, carbon and nitrogen source using BBD affected the enzyme production. Different runs correspond to variation in the enzyme production. A significant inter-correlation between the observed and predicted data represents the significance of the model. The determination coefficients correspond to the difference between the observed and predicted data. 3-D response graphs show the interaction between two independent variables. 3D response plot predicts that the optimum region for enzyme production is at more or less at central values of the parameters.

Conclusions

Xylose transport mechanism and key enzymes are involved in the xylitol production. It led to the search for the production of XR from various sources. On the other hand, the production of enzyme was also enhanced in shorter time interval by RSM using BBD. The fermentation conditions such as inoculum (1%), HWs corncob (Carbon source) (0.5% w/v), time period (60) and RPM (140) showed maximum production of enzyme. The experimental data showed close agreement with predicted values under optimized conditions, confirming the validity of this model. The optimized model using BBD showed 3.7 fold increments in XR production compared to OFAT method. The designed model based upon the multi-variable analysis provides the strategy to produce enzyme from this particular isolate at pilot scale using fermenter. Further study is in progress to produce xylitol using different hemicellulosic waste substrates and to express xylitol producing gene of *C. tropicalis* strain LY15 into *Saccharomyces* species through cloning strategies.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Aguiar WB, Faria LFF, Couto MAPG, Araujo OQF, Pereira N (2002). Growth model and prediction of oxygen transfer rate for xylitol production from d-xylose by *C. guilliermondii*. Biochem. Eng. J. 12:49-59.

Altamirano A, Vazquez F, De Figueroa LIC (2000). Isolation and identification of xylitol producing yeasts from agricultural residues. Folia Microbiol. 45: 255-258.

Alvira P, Negro MJ, Ballesteros I, González A, Ballesteros M (2016).

Steam explosion for wheat straw pretreatment for sugars production. Bioethanol. 2(1).
Anne Usvalampi (2013). Microbial production of xylitol, l -xylulose and l-xylose. Doctoral thesis. Aalto University publication series, Finland.
Aro N, Pakula T, Penttila M (2005). Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiol. Rev. 29:719-739.
Ayadi I, Kamoun O, Trigui-Lahiani H, Hdiji A, Gargouri A, Belghith H, Guerfali M (2016). Single cell oil production from a newly isolated *Candida viswanathii* Y-E4 and agro-industrial by-products valorization. J. Ind. Microbiol. Biotechnol. 43:901-914.
Barathikannan K, Agastian P (2016). Xylitol: Production, Optimization and Industrial Application. Int. J. Curr. Microbiol. Appl. Sci. 5(9):324-339.
Barathikannan K, Khusro A, Agastian P (2016). Isolation and molecular characterization of xylitol producing wild yeast strains from different fermented fruit juices. South Indian J. Biol. Sci. 2(4):415-423.
Converti A, Dominguez JM (2001). Influence of Temperature and pH on xylitol Production from xylose by *Debaryomyces hansenii*. Biotechnol. Bioeng. 75:39-45.
Dominguez JM, Gong CS, Tsao GT (1997). Production of Xylitol from D-Xylose by *Debaryomyces hansenii*. Appl. Biochem. Biotechnol. 63:117-127.
Fredlund E, Druvefors U, Boysen EM, Lingsten K, Schnurer J (2002). Physiological characteristics of the biocontrol yeast Pichia anomala J121. FEMS Yeast Res. 2:395-402.
Guo C, Zhao C, He P, Lu D, Shen A, Jiang N (2006). Screening and characterization of yeasts for xylitol Production. J. Appl. Microbiol. 101:1096-1104.
Hallborn J, Walfridsson M, Airaksinen U, Ojamo H, Hahn-Hagerdal B, Penttila M, Kerasnen S (1991). Xylitol production by recombinant *Saccharomyces cerevisiae*. Biotechnol. 9:1090-1095.
Heard GM, Fleet GH (1985). Growth of natural yeast flora during the fermentation of inoculated wines. Appl Environ Microbiol. 50:727-728.
Izumori K, Tuzaki K (1998). Production of xylitol from D-xylulose by *Mycobacterium smegmatis*. J. Ferment. Technol. 66:33-36.
Khusro A, Barathi Kannan K, Al-Dhabi NA, Arasu MV, Agastian P (2016). Statistical optimization of thermo-alkali stable xylanase production from *Bacillus tequilensis* strain ARMATI. Electron J. Biotechnol. 22:16-25.
Kim JH, Han KC, Koh YH, Ryu YW, Seo JH (2002). Optimization of fed-batch fermentation for xylitol production by *Candida tropicalis*. J. Ind. Microbiol. Biotechnol. 29:16-29.
Lin Y, He P, Wang Q, Lu D, Li Z, Wu C, Jiang N (2010). The alcohol dehydrogenase system in the xylose-fermenting yeast *Candida maltosa*. PLoS ONE 5:e11752.
Lopez F, Delgado OD, Martinez MA, Spencer JF, Figueroa LI (2004). Characterization of a new xylitol-producer *Candida tropicalis* isolates. A Van Leeuw J. Microb. 85:281-286.
Margolles-Clark E, Ilmaon M, Penttilla M (1997). Expression patterns of ten hemicellulase genes of the filamentous fungus *Trichoderma reesei* on various carbon sources. J. Biotechnol. 57:167-179.
Misra S, Raghuwanshi S, Saxena RK (2013). Evaluation of corncob hemicellulosic hydrolysate for xylitol production by adapted strain of *Candida tropicalis*. Carbohydr. Polym. 92:1596-601.
Moyses DN, Reis VC, Almeida JR, Moraes LM, Torres FA (2016). Xylose Fermentation by Saccharomyces cerevisiae: challenges and prospects. Int. J. Mol. Sci. 17:207.
Mussatto SI, Roberto IC, (2004). Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. Bioresour. Technol. 93(1):1-10.
Oh DK, Kim SY, Kim JH (1998). Increase of xylitol production rate by controlling redox potential in *Candida parapsilosis*. Biotechnol. Bioeng. 58:440-444.
Paidimuddala B, Gummadi SN (2014). Bioconversion of Non-Detoxified Hemicellulose Hydrolysates to Xylitol by Halotolerant Yeast *Debaryomyces nepalensis* NCYC 3413. J. Microb. Biochem. Technol. 6:327-333.
Parajo JC, Dominguez H, Domi nguez JM (1998). Biotechnological production of xylitol. Part1: Interest of xylitol and fundamentals of its biosynthesis. Bioresour. Technol. 65(3):191-201.
Pérez J, Muñoz-Dorado J, de la Rubia RT, Martínez J (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int. Microbiol. 5:53-63.
Pscheidt B, Glieder A (2008). Yeast cell factories for fine chemical and API production. Microb. Cell Fact. 7:25.
Rafiqul ISM, Sakinah AMM (2015). Biochemical properties of xylose reductase prepared from adapted strain of *Candida tropicalis*. Appl Biochem. Biotechnol. 175:387-399.
Rangaswamy S, Agblevor FA (2002). Screening of facultative anaerobic bacteria utilizing d-xylose for xylitol production. Appl. Microbiol. Biotechnol. 60:88-93.
Rengaraju B, Ponnuswamy R, Lakshminarayanan V (2014). Investigation on *Candida Parapsilosis* strain BKR1 for the Production of Xylose reductase enzyme and its Kinetic Parameter Appraisal. Int. J. Chem. Tech. Res. 6:4803-4806.
Rodrigues RC, Felipe MG, Roberto IC, Vitolo M (2003). Batch xylitol production by *Candida guilliermondii* FTI 20037 from sugarcane bagasse hemicellulosic hydrolyzate at controlled pH values. Bioprocess Biosyst. Eng. 26:103-107.
Saha B, Bothast R (1999). Production of xylitol by *Candida peltata*. J Ind. Microbiol. Biotechnol. 22:633-636.
Saha BC, Bothast RJ (1997). Microbial production of xylitol. In BC Saha, J Woodward, eds, Fuels and Chemicals from Biomass. American Chemical Society, Washington, DC. pp. 307-319.
Silva SS, Felipe MG, Mancilha IM (1998). Factors that affect the biosynthesis of xylitol by xylose-fermenting yeasts. A review. Appl. Biochem. Biotechnol. 70:331-339.
Tamburini E, Costa S, Marchetti MG, Pedrini P (2015). Optimized Production of Xylitol from Xylose Using a Hyper-Acidophilic *Candida tropicalis*. Biomolecules. 5(3):1979-1989.
Vandeska E, Amartey S, Kuzmanova S, Jeffries TW (1995). Effects of environmental conditions on production of xylitol by *Candida boidinii*. World J. Microbiol. Biotechnol. 11:213-218.
Winkelhausen E, Kuzmanova S (1998). Microbial conversion of d-xylose to xylitol. J. Ferment. Bioeng. 86:1-14.
Zagustina NA, Rodionova NA, Mestechkina NM, Shcherbukhin VD, Bezborodov AM (2001). Formation of xylitol in *Candida guilliermondii* 2581 culture. Prikl. Biokhim. Mikrobiol. 37:573-577.

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Effects of acidification on the extension of shelf life of Japanese wet-type noodles (woo-long noodle)

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Noodles are one of the most important foods worldwide; various methods have been reported to extend their shelf life. In this study, the effects of dipping noodles in various concentrations of acidulant solutions (organic acids) on the shelf life of the noodles were evaluated. The results showed that the growth of the tested microorganisms, that is, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*, was significantly inhibited when > 0.5% solutions of acidulants were applied. Here, a 0.5% mixed solution showed a larger halo zone diameter (8.5 to 11.5 nm) than 0.5% citric acid or 0.5% lactic acid solution (8.0 to 9.0 nm). Moreover, six variations of acidulants were used (0.5 and 1% citric acid, lactic acid and their combination), the shelf life at 5°C of the noodles dipped in a 0.5% 1:1 mixed solution for 20 s was also 4 to 6 days longer than those without treatment. All sensory attributes of noodles treated with the mixed solution were the highest ($p<0.05$) among all the samples. The results of the sensory study also indicated that products treated with the mixed solution had scores that were close to those of untreated noodles, in terms of appearance, texture, taste, hardness and elasticity as well as overall acceptance. Finally, these results provide support for developing a simple method to prolong the shelf-life of fresh noodles and minimizing any negative changes in sensory attributes.

Key words: Acidity, organic acid, antimicrobial agents, microorganisms, sensory evaluation

INTRODUCTION

Noodles are one of the most important foods in Asian cuisine, and the flour used to make noodles in Asia accounts for approximately 40% of the total flour consumed there (Hou and Kruk, 1998). In addition to wheat flour, noodles are made from simple ingredients such as water, salt or alkaline salt and contain carbohydrates, protein and small amounts of fatty acids. Wet-type noodles, such as Chinese yellow alkaline noodle and Japanese white woo-long noodle, are very popular in local market due to their unique mouth-feel and convenience for eating. The most common spoilage organisms in such fresh noodles are bacteria, followed by

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yeast and moulds (Ray, 2001). Due to their high water content, high pH and Aw value, wet raw noodles have a short shelf-life because of microbial spoilage and browning reaction. However, the main problem is that the short shelf life of noodles results in high levels of waste in the industry and may also be a potential source of food poisoning (Ghaffars et al., 2009). Yellow alkaline noodles are partially boiled wet noodles with a pH range of 9.0 to 10.0. Microbial or fungal growth causes the development of sliminess on the surface of noodles at this pH range (Karim, 1989), and thus these noodles are very susceptible to spoilage and have a shelf life of 1 to 2 days. Spoilage is not only due to the visible growth of microorganisms, but also to the production of end metabolites which cause off-odours and slime production (Forsythe, 2000).

In recent years, various methods have been reported to extend the shelf life of noodle products such as free additives. The products are called semi-dried noodles, which ensure both the similar flavour and taste to fresh noodles and a prolonged shelf-life (Li et al., 2016). Irradiation by 10 kGy of 60 Co gamma rays increased the shelf life of fresh noodles to up to 10 days when stored at room temperature (Jianming, 1998). Fu et al. (2008) noted that fresh noodles with neutral pH made using a food grade monolaurin microemulsion system (MMEs) had a shelf life of up to 10 days when stored at 37°C. The addition of *Brevibacterium helvolum* B8 and *Arthrobacter* sp. was attempted as a biological method to increase shelf life; B8 improved the quality of yellow alkaline noodles and extended the shelf life to 7 days (Saito, 2003). Natural antibacterial compounds from lemon extracts and traditional herbs and spices may potentially be useful as food preservatives to improve the microbiological stability of fresh pasta and noodles (Li et al., 2014).

Organic acids, such as citric, malic, acetic, fumaric, tartaric, adipic, lactic, ascorbic acid, etc. have a long history of use as food additives and preservatives for preventing food deterioration and extend the shelf life of foods (Howard et al., 1994). However, little literatures investigated the effects of acidification on the shelf life of noodle products. Many researchers have used organic acid solutions in combination with heat treatment (pasteurization) to increase the shelf life of fresh pasta and noodles (O'Rourke et al., 2003). McGuire et al. (1989) applied hurdle technology, a combination of dough pasteurization, modified atmosphere packaging and chilling to preserve fresh pasta and succeeded in extending the shelf life to 120 days. Li et al. (2011) investigated the effect of water activity, irradiation and their combination on the shelf-life of fresh noodles. It could be extended for more than 7 times by reducing Aw from 0.979 (control) to 0.900 with a combination of humectants including glycerol, propylene glycol, compound phosphate and salt.

To develop a simple method to prolong the shelf-life of fresh noodles and minimize any negative changes in sensory attributes, the present study was therefore, undertaken to determine the best conditions for acidification of woo-long noodles. The effects of dipping noodles in various concentrations of acidulant solutions on the shelf life of the noodles were determined.

MATERIALS AND METHODS

Antibacterial assay

The antibacterial activity of the acidulant solutions was determined according to the methods described by Piddock(1990) with some modifications. The tested microorganisms for the bioassay were *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (BCRC 12606), *Bacillus cereus* (BCRC 14196), which were grown on DC agar or TSA agar or NA agar plates, respectively at 35°C overnight. These plates are referred to as seeded agar. Sensitivity discs (Gram-positive and negative) were used to assay the sensitivity pattern. Eighty microliters of various concentrations of lactic acid and citric acid solution were aseptically pipetted onto the paper discs (Diameter: 8 mm, Advantec), which were placed on individuals plates at 35°C for 24 h. The sensitivity of the tested microorganisms to the acids was indicated by a halo around the discs where no bacterial growth occurred. The diameter of the halo was taken as an index of the degree of sensitivity and was measured with a transparent plastic ruler.

Preparation of noodles

An HK-201 mixer (Hoqurt Co., Ltd.) was used to mix the noodles ingredients and increase the amount of gluten (Moss et al., 1987). The noodle formulation used for all experiments consisted of 100 parts flour, 35 parts water (based on a flour moisture of 11.3%) and 1 part sodium chloride. Sodium chloride was dissolved in the water and added to 300 g of flour in the mixer over 2 min on the slow speed mixing (setting 1). Mixing was continued for 2 min at high speed (setting 2), followed by an additional mixing for 1 min at slow speed. The ingredients were passed through a Chung Hou laboratory noodle machine (Chung Hou Bakery Machine Co., Ltd.) with a gap setting of 1.0 mm. The sheet was folded in two and passed through rollers twice more. The dough was allowed to rest at room temperature in a plastic container for 30 min. The noodle dough was obtained by kneading with conventional roll sheeting and other methods, and was formed into a noodle sheet by sheeting or sheeting/combining. The noodles sheet was folded between passes to ensure its uniformity (Oh et al., 1985). The thickness of the noodle sheet after subsequent maturing between 3 and 6 mm, preferably 5 mm for woo-long noodles (standard noodles) to ensure good viscoelasticity, smoothness, taste and other characteristics (Keiithiro, 2001). The sheet was then cut into noodle strands quickly by pasta machine. To prevent the noodles from sticking together, they were usually coated with a thin layer of wheat flour. The noodles were pre-cooked in boiling water for 2.5 min, with a ratio of at least 1:10 noodles to water (Anon, 1985). The partially cooked noodles were subsequently rinsed with cool tap water and were then ready for further testing.

Process for acidification of noodles

After cooling, the partially cooked noodles were immersed in ten times weight of various concentration of citric acid or lactic acid solution, that is, 0.1% CA or LA, 0.25% CA or LA, 0.5% CA or LA, 1.0% CA or LA, at RT for 20 s. The physical, chemical, cooking and

sensory properties of all treated noodles were analysed.

Chilled storage test

The experimental batches of woo-long noodles were stored at chilled storage (5°C). Storage was continued for 10 days and microbiological testing was performed for each treatment after every day of storage. In addition to estimation of the counts of the tested microorganisms, the total aerobic viable cell counts, were estimated from appropriate dilutions prepared from duplicate packages for each treatment. Total aerobic plate counts were estimated in PCA (casein-peptone-dextrose-yeast agar, HiMedia Laboratories, M091S-500G) after 30°C incubation for 2 to 3 days. In each experiment, two independent packages were tested individually for their microbiological status. The mean values of log colony counts from duplicate plates of relevant dilutions were then given in the tables or plotted in figures.

Analysis of cooking characteristics

Cooking loss

Cooking loss (%) was calculated as described by Lee and others (1998) with some modifications (AACC, 2000; Lee et al., 1998). Distilled water (500 mL) in a beaker was heated on a hotplate until it started boiling. Samples of partial cooked noodles (50 g) were put in the beaker. The noodles were cooked in boiling water for 3 min. The cooking water was poured into a 250-mL volumetric flask; the final volume (500 mL) was then topped off with distilled water. The volumetric flask was shaken to homogenize the cooking water solution. A 20 mL aliquot of the solution was pipette into an aluminum dish, and the sample was dried in an oven at 105°C until a constant weight was obtained. The cooking loss was measured by the following equation below: cooking loss (%) = [dried residue in cooking water (g)/noodle weight before cooking (g)] × 100.

Colour and pH Value

The noodles were analysed for moisture, protein, fat and ash content using standard procedures AOAC (2000). The colour analysis of the partially cooked noodles was performed using a TC-1500 DX spectrophotometer (Tokyo Denshoku, Japan) at ambient temperature. The colorimeter was calibrated by using white and black standards. The colour values L∗, a∗ and b∗ were measured with a C illuminant and a 10° standard observer. The dimension L∗ indicates lightness, with 100 for white and 0 for black; the a∗value indicates redness when positive and greenness when negative; and the b∗value represents yellowness when positive and blueness when negative. The white index (WI) was measured by the following equation: $WI = 100\sqrt{(100-L)^2+a^2+b^2}$. The pH of a cooked noodle slurry was measured using a microcomputer pH-VISION 6071 pH meter (JENCO electronics, LTD, NY, USA), which was calibrated using buffered solutions of pH 4.0 and 7.0 (Ng et al., 2011).

Texture analysis

Noodle firmness was measured according to the methods described by Bourne (1978) with some modifications. A TA.XT2 Texture Analyser (Stable Micro System, Surrey, United Kingdom) with 25-kg load cell was used to measure the firmness of cooked noodles. On the basis of preliminary trials, texture parameters were set at pretest speed = 1.0 mm/s; test speed = 1.0 mm/s; post-test speed = 1.0 mm/s; strain 50%; trigger force Auto-5g; data Acquisition = 200 pps; and adapter No.10. Noodle samples were evaluated within 5 min after cooking. Five strands of cooked noodles were placed parallel to each other on a flat plastic plate. The tensile test was performed, and twelve measurements for each sample were collected.

Shearing force

Five noodle strands with or without acidification were randomly selected and their shearing force was determined with a Sun Rheometer (Model CR-200D, Sun Scientific Co., Ltd. Japan). The parameters were set as follows: test speed: 6 cm/min; compression distance: 15 mm and adapter: No. 10. Twelve data readings were collected for each noodle treatment and all of the data were used in the statistical analysis.

Sensory evaluation

The noodle samples were boiled and cut into 8 cm pieces. The samples weighted 15 to 20 g for each were stored in tightly covered plastic containers, which were kept in a food warmer before testing. The sensory attributes of the cooked noodles were evaluated by 30 panelists consisting of students of the Department of food Technology and Marketing Management, Taipei College of Maritime Technology. All samples were evaluated using a five point hedonic scale, where ‘‘1” indicated ‘‘dislike very much” and ‘‘5” indicated ‘‘like very much”. Each noodle sample was coded with a random set of 3 numbers. The panelists were given drinking water to rinse their mouths before evaluating each sample. The panelists evaluated intensity of noodle colour, roughness, stickiness, firmness, taste, aftertaste, elasticity and flavour.

Statistical analysis

All noodle sample data were analysed with SAS computer software (SAS 2002–2003). An analysis of variance (ANOVA) was conducted to determine the significance of the difference among each of the 3 treatments. Any value that was considered significantly different ($P<0.05$) was subjected to Duncan’s multiple range test. The colour and textural characteristics of the noodles were determined at least in triplicate in replicate samples.

RESULTS AND DISCUSSION

The antibacterial activity of various concentrations of CA and LA solutions were examined and judged by assaying the patterns of bacterial sensitivity. Table 1 summarizes the halo zone test results for the tested microorganisms. Based on the diameter of the halo zone, both LA and CA had similar effect on Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and negative bacteria (*E. coli*) tested.

It was found that the combination of CA and LA was more effective, with zones of inhibition ranging from 9.5 to 21.0 mm. The antimicrobial activity of CA and LA against *B. cereus* was slightly lower than against *E. coli* and *S. aureus.* Higher concentration or the combination of CA and LA likely contributed at least in part to the generation of a larger inhibition zone. The mechanism of

Table 1. Inhibitory effect of various concentrations of CA and LA on *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus.*

Test strain	Acid	Concentration (%)			
		0.5	1	3	5
E. coli	CA*	8.5**	10	12	16
	LA	8.5	9	13	15
	CA & LA	9.5	10	13.5	16.5
S. aureus	CA	8	8.5	9.5	16
	LA	8.5	11	12	15
	CA & LA	8.5	9.5	13.5	17.5
B. cereus	CA	9.5	11	17	20
	LA	9	9.5	15	18.5
	CA & LA	11.5	14	18.5	21

* CA: Citric acid; LA; lactic acid; CA & LA: citric and lactic acid (1:1); ** The diameter of the inhibition zone (mm).

Table 2. Cooking loss of the acidified woo-long noodles.

Acid	Concentration (%)				
	0	0.1	0.25	0.5	1
CA*	4.47^{g}** ± 0.21	5.89^{def} ± 0.30	6.19^{cde} ± 0.23	8.31^{a} ± 0.68	8.48^{a} ± 0.25
LA	4.47^{g} ± 0.21	5.86^{def} ± 0.30	6.16^{cde} ± 0.23	7.43^{b} ± 0.55	7.48^{b} ± 0.23
CA & LA	4.47^{g} ± 0.21	5.26^{fg} ± 0.56	5.55^{ef} ± 0.48	6.85^{bc} ± 0.21	6.70^{bcd} ± 0.23

*CA: Citric acid; LA: lactic acid; CA and LA: citric acid and lactic acid (1:1); **$^{a-g}$ Mean values with different letters are significantly different ($p<0.05$); ***Results are mean ±SD of three determinations.

the antimicrobial action of organic acids is not fully understood yet, and activity may vary depending on physiological status of the organism and the physicochemical characteristics of the external environment (Ricke, 2003). Organic acids such as lactic acid are known to inhibit the cell growth and the active uptake of some amino and keto acids into bacterial membrane vesicles in *E. coli* and *B. subtilis* (Doores, 1993). The combination of CA and LA at a final concentration of 0.5% showed a larger halo zone diameter than the same concentration of CA or LA alone, and the diameter of the growth inhibitory zone for CA and LA against *B. cereus* was slightly larger than that against *E. coli* and *S. aureus.*

The proximate composition of the noodles with or without acidification, such as the moisture, protein, fat, ash and carbohydrate contents, were similar to that of commercially available wet noodles in Taiwan in terms of the protein (11.3%), ash (0.4%) and carbohydrate (87.7%) contents. The typical pH value of woo-long noodle from domestic markets in Taipei, Taiwan ranges between 4.12 and 6.58 (marketing survey in this study, data not shown). The pH of the noodles was reduced from 5.79 to 3.84 when treated with different concentrations of acid solutions. The higher the concentration of acidulants added to the sample, the more the pH value is reduced (data not shown).

According to Shiau and Yeh (2001), the higher the cooking loss, the stickier the noodle surface. Serious cooking loss is undesirable because it means that there was a high starch content in the cooking medium and that the noodles had a low cooking tolerance (Chakraborty et al., 2003). As shown in Table 2, the cooking loss of acidified noodles in this study showed a significant increase ($P<0.05$) when the concentration of the acid used for the noodle treatment increased. This is especially true for the case of citric acid-treated samples. This result might be due to low dissociation constant of citric acid (Malyniak and Meagher, 1990). Acidification of noodles with 1% CA or LA may affect the sensory properties and texture of the cooked product based on the results of this study.

Shiau and Yeh (2001) indicated that starch gelatinized at 80°C, when its viscosity, elasticity, cutting force and tensile strength increased at high temperature. Once the noodles were cooked, starch gelatinization occurred on the surface of the noodles, contributing to a greater cooking loss (Shiau, 1996). However, the mixture of both

Table 3. Effects of different acid treatments on the colour (L, a, b) and WI values of woo-long noodles.

Acid	Concentration (%)	Colour			
		L	a	b	WI
CA*	0	55.04±1.43^{cd}	− 1.05±0.06^{abc}	10.29±0.4^{bc}	54.85±1.02^{bcd}
	0.10	54.62±1^{d}	− 1.04±0.05^{abc}	11.57±1.63^{a}	53.13±0.99^{c}
	0.25	54.65±1.33^{d}	− 1.04±0.04^{abc}	10.58±1.34^{ab}	53.4±1.24^{c}
	0.50	54.71±0.58^{d}	− 1.1±0.07^{c}	10.1±1.01^{cd}	53.58±0.55^{c}
	1	55.81±1.15^{abc}	− 1.05±0.09^{c}	10.01±1.18^{bcd}	54.66±0.97^{bcd}
LA*	0	55.04±1.43^{cd}	− 1.05±0.06^{abc}	10.29±0.4^{bc}	54.85±1.02^{bcd}
	0.10	55.11±1.06^{cd}	− 1±0.07^{ab}	9.88±0.84^{bcd}	54.17±1.1^{cde}
	0.25	55.13±0.65^{cd}	− 1.09±0.05^{bc}	10.2±0.51^{c}	53.97±0.6^{de}
	0.50	56.25±0.62^{ab}	− 0.99±0.04^{a}	9.12±0.66^{cdef}	55.13±0.74^{abc}
	1	56.52±0.57^{a}	− 1.05±0.09^{abc}	7.81±0.7^{g}	55.82±0.5^{a}
CA and LA*	0	55.04±1.43^{cd}	− 1.05±0.06^{abc}	10.29±0.4^{bc}	54.85±1.02^{bcd}
	0.10	55.38±0.75^{bcd}	− 1.06±0.06^{abc}	10.56±0.99^{ab}	54.13±0.72^{cde}
	0.25	56.35±0.86^{ab}	− 1.06±0.03^{abc}	8.67±0.65^{efg}	55.48±0.94^{ab}
	0.50	56.58±0.82^{a}	− 1±0.11^{ab}	8.47±0.87^{fg}	55.74±0.88^{a}
	1	56.23±0.59^{ab}	− 1±0.06^{ab}	8.81±1.03d^{efg}	55.34±0.47^{ab}

* CA: Citric acid; LA: lactic acid; CA and LA: citric acid and lactic acid (1:1); **Values in the same column with significantly different ($p<0.05$). ***Results are mean ±SD of three determinations.

CA and LA in equal amounts resulted in lower cooking lost (Table 2). The amount of residue in the cooking water is commonly used as an indicator of the quality of cooked spaghetti and low amounts of residue indicate high pasta cooking quality (Del Nobile et al., 2005). Dick and Youngs (1988) considered cooking loss of 7 to 8% to be acceptable for dried pasta. Additionally, when compared with dried pasta, in this study, cooking losses for all noodles treatments were <8%.

The appearance of noodles is important to consumers, and the colour of the noodles is the most vital quality parameter. The colour of uncooked noodles should be bright and yellow without specks. The colour of the partially cooked noodles with or without acidification was analysed, and the colour was roughly distinguished by eye. The colour of the boiled noodles with or without acidification is presented in Table 3. Their lightness values (L*) was not very different. Beside citric acid, the noodles treated by 0.1 to 1.0% of lactic acid or both have a lightness value (L*) higher than controls, similar to the results described by Shiau (1996). Lactic acid additives can increase lightness values of noodles, and they also show negative values for redness (a*) and low positive values for b*. Decreases in these values as the concentration of the acid use increases are attributed to the presence of natural flavonoid pigments, which are colourless at acidic pH levels but turn yellow at alkaline pH levels (Shelke et al., 1990). As seen in Table 3, the lightness (L*), yellowness (a*), redness (b*) and white index (WI) did not change significantly ($p<0.05$), indicating the exterior stability of the noodles. The results also shows that the noodles treated by 0.5% CA & LA has good lightness (L*) and WI values.

In contrast to colour, noodle texture characteristics are more complicated and less understood. The texture of cooked noodles is considered the most critical characteristic in evaluating the quality of noodles and determining consumer acceptance of the product (Bhattacharya et al., 1999). Tensile strength represents the consumption quality of noodles, and it also corresponds to elasticity of the noodles (Chakraborty et al., 2003).

The Japanese preference for woo-long noodle is for a softer, slightly more elastic and adhesive texture. When noodles are cooked, the Japanese woo-long noodle has a significantly different cutting hardness and cohesiveness than Chinese wheat flour noodles, which could be caused by the differences in the protein and carbohydrate composition of the noodles. It is highly likely that woo-long noodle flour is lower in amylase as compared to the Chinese flour. The insoluble glutenin to total protein ratio of Japanese woo-long noodle is higher than the Chinese wheat varieties (Hu et al., 2007).

In this study, the differences in the cooking quality of the noodle after treatments can be attributed to the differences in the acid level of the treatments. Higher concentrations of acidulants significantly decreased ($p<0.05$) the shearing force of the noodles, as shown in

Table 4. Effects of different acid treatments on the shearing force values of woo-long noodles (n=12).

Acid	Shearing force (mm^2/g)				
	0%	0.10%	0.25%	0.50%	1%
CA	598±70[a]	496±89[ab]	528±77[ab]	473±75[bc]	386±95[c]
LA	598±70[a]	595±39[ab]	589±84[ab]	534±72[ab]	500±57[bc]
CA+LA	598±70[a]	586±89[ab]	581±75[ab]	562±57[ab]	531±39[bc]

*CA: Citric acid; LA: lactic acid; CA and LA: citric acid and lactic acid (1:1); **Results are mean ±SD of three determinations.

Table 4. This was particularly obvious for noodles immersed in citric acid. This result might be due to the action of hydrogen ions or anionic acid residues that cleave the salt linkages of protein molecules, resulting in softer and weaker internal structures as well as molecular binding. Such cleavage might have weakened the protein gluten network as well as the hydrogen binding of the molecules (Tanaka et al., 1967). On the other hand, the increased stickiness and higher cooking loss are likely caused by the diffusion of amylose out from the noodle surface (Shiau and Yeh, 2001).

Commercially available noodle products (domestic market) in Taipei, Taiwan were collected and employed in this study. Microbiological examinations of wet noodles sold in the market showed that woo-long noodles without packaging could be stored for 1 day at 20°C and 3 days at 5°C, respectively. Noodles that were packed and then pasteurized could be stored for 2 to 3 days at 20°C (data not shown). However, depending on the heat resistance of the packaging materials used, products could be stored for 9 days or 1 month longer at 5°C. In terms of woo-long noodles, those subjected to acidification, vacuum packaging and pasteurization at high temperature could be stored at least 3 months with no microbial growth or change in pH value for products at either 5 or 20°C (unpublished data). Samples prepared in the laboratory were evaluated by the chilled storage test. The experimental batches of woo-long noodles were stored at chilled storage at 5°C. Samples of woo-long noodle before and after acidification had similar moisture contents (66.7% and 65.0-68.0%, respectively) and Aw (0.98-0.99) (unpublished data).

As mentioned earlier, the noodles with or without acidification had similar water activities and moisture contents during refrigerated storage with or without acidification. Low pH seemed to impart a bacteriostatic effect on the noodles. In this study, 6 log cfu/g of APC was considered the microbiological quality limit, beyond which the product would be unsuitable for consumption, which is consistent with the limit used by Lee et al. (2001). On any given storage day, the acidified samples had lower APC than the control group. The control, 0.1% CA & LA and 0.25% CA & LA groups exceeded 6 log cfu/g of APC by day 4 (Figure 1). The 0.5% CA and 0.5% LA groups were within this limit after 8 days but exceeded the limit by 10 days (Figure 1). The 0.5% CA & LA and 1% CA & LA groups did not exceed 6 log cfu/g of APC by 10 days. Thus, in this study, the acidification of noodles (0.5% CA and LA, and 1% CA and LA) increased theshelf life of noodles from 4 to 10 days. During storage at 5°C, the moisture content of both samples (0.5% CA and LA, and 1% CA and LA) declined slowly, and water activity was similar for both samples. Small changes in pH occurred over the 10 days storage, but overall, the changes in pH were very small. Changes in pH can occur in food products as a result of microorganism activity and other chemical reactions. Some microorganisms, such as lactic acid bacteria, reduce the pH by producing acids, and some proteolytic bacteria and mold increase the pH by releasing ammonia from proteins.

The evaluation of the sensory attributes of cooked noodles with or without acidification by a trained panel is shown in Table 5. The sensory evaluation results showed that there were statistically insignificant differences ($p > 0.05$) in the colour, hardness and elasticity among the 0.1, 0.25 (unpublished data), 0.5% and control (0%) groups (Table 5).

The panelists failed to differentiate between the colour of the control and 0.5% (CA + LA). A significant reduction ($p<0.05$) was also noted in the taste, aftertaste and overall acceptance of the noodles when acidified with 0.5% (CA+LA). All sensory attributes of 0.5% (CA+LA) noodles were rated the best ($p<0.05$) among all samples; the 1% CA, 1% LA and 1% (CA+ LA) noodles showed the lowest score among all samples in all sensory attributes in the appearance and texture categories. The higher the concentration of CA or LA that was added to the noodles, the less acceptable the noodles were to the panelists. The increase in the acid concentration (1% CA and 1% LA) might have contributed a more sour taste and aftertaste to the noodles as well as to a softer and less elastic texture; these characteristics might not have been favourable to the panelists.

The 0.5% CA treated noodle texture received a viscoelasticity score of 3.18 and smoothness score of 3.32, which were less preferred than the 0.5% (CA + LA) noodles (viscoelasticity score=3.82 and smoothness score=3.64). The overall acceptance of the noodles was in the following order: 1% CA<1% LA<0.5% CA<0.5% LA<0.5% (CA + LA). Generally, acidification by LA may cause a sour, astringent taste. The 0.5% LA noodles received a flavour score of 3.06, so they were less

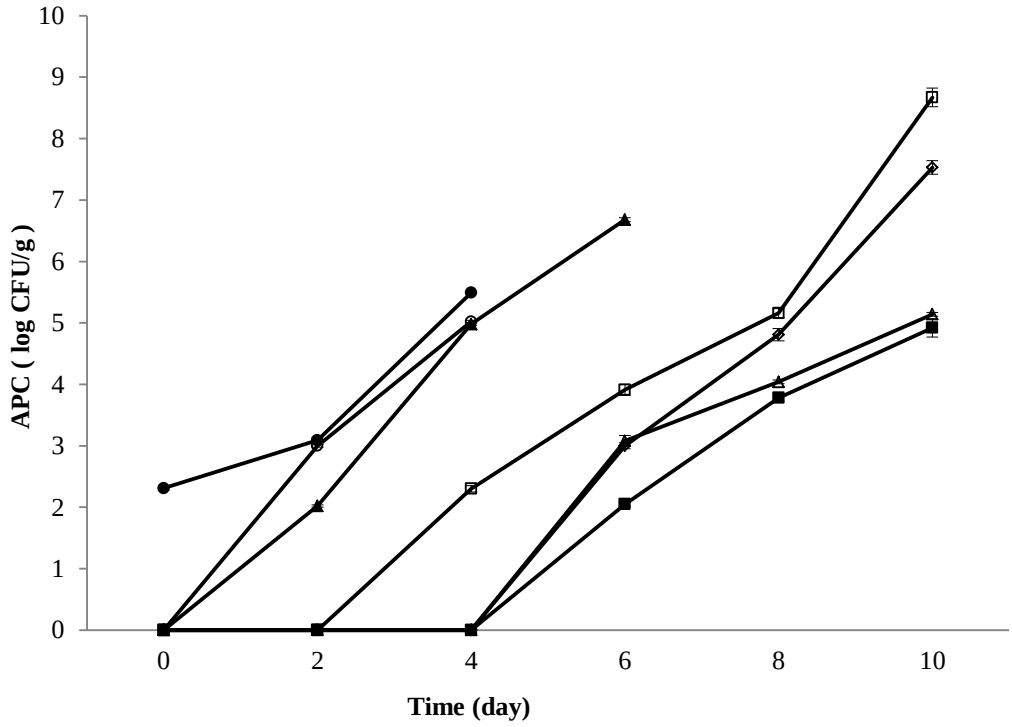

Figure 1. Effects of various combinations of CA and LA on the total bacterial counts of noodles during storage. ●: Control; ○: 0.1% CA & LA; ▲: 0.25% CA & LA; △: 0.5% CA & LA; ■: 1% CA & LA; □: 0.5% CA; ◇: 0.5% LA. Results are mean ±SD of three determinations.

Table 5. Perception of woo-long noodles subjected to different acid treatments.

Acid	Appearance		Texture			Flavour	Total
	Colour	Surface appeal	Hardness	Viscoelasticity	Smoothness		
None	4.32^{a**}±0.65	3.90^{a}±0.81	3.59^{a}±0.73	3.77^{a}±0.75	3.59^{a}±0.792	3.50^{a}±0.74	22.45^{a}±3.09
0.5% CA*	4.09^{a}±0.64	3.59^{a}±0.79	3.36^{a}±1.14	3.18^{b}±0.96	3.32^{a}±0.89	3.50^{a}±0.51	21.05^{a}±3.169
1% CA	2.20^{c}±0.45	3.20^{a}±1.64	2.60^{b}±1.07	2.60^{b}±1.14	2.20^{b}±1.30	1.60^{b}±0.55	14.40^{b}±5.97
0.5% LA	4.05^{a}±0.72	3.68^{a}±0.89	3.32^{a}±1.08	3.41^{ab}±1.05	3.32^{a}±1.00	3.06^{b}±0.66	21.13^{a}±3.15
1% LA	2.40^{c}±0.89	3.20^{a}±1.10	2.80^{b}±1.41	2.80^{b}±1.00	2.60^{b}±1.34	1.40^{b}±1.67	15.20^{b}±5.02
0.5% CA+ LA	3.91^{a}±0.61	3.59^{a}±0.67	3.64^{a}±0.73	3.82^{a}±0.92	3.64^{a}±0.66	3.50^{a}±0.51	22.05^{a}±2.68
1% CA+ LA	3.00^{bc}±0.71	3.00^{a}±0.71	2.92^{b}±0.84	2.40^{b}±0.89	2.60^{b}±1.14	2.30^{b}±1.00	16.32^{b}±3.56

*CAL Citric acid; LA: lactic acid; CA and LA: citric acid and lactic acid (1:1); **Values in the same column with different letters are significantly different ($p<0.05$). ***Results are mean ±SD of three determinations.

preferred than the 0.5% CA and 0.5% (CA+ LA) noodles (flavours score 3.50 for both groups). The reduction in the overall acceptability of noodles of 0.5% CA, 0.5% LA and 0.5% (CA + LA) noodles as compared to the control was 21.05, 21.13, 22.05 and 22.45%, respectively. The overall acceptance data showed that the noodles treated with 0.5% (CA + LA) was still accepted by panelists.

Conclusions

In this study, it was found that noodle acidification by immersing in ten times weight of citric acid or lactic acid solution for 20 s could improve the shelf life of noodles. As a result, a 0.5% mixture of solution of 1:1 CA and LA resulted in 4 to 6 days longer shelf life than noodles without treatment. Furthermore, the noodles showed a trend that had statistically insignificant differences ($p >0.05$) in the colour, hardness and elasticity among the 0.1, 0.25, 0.5% and control (0%) groups. Moreover, a significant reduction ($p<0.05$) was also noted in the taste, aftertaste and overall acceptance of the noodles when acidified with 0.5% (CA+LA). However, further studies need to be carried out to determine whether hurdle

technology, using a combination of pasteurization, modified atmosphere packaging and chilling to preserve fresh noodles, could also help to minimize changes of sensory attributes during a long storage period.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Anon (1985) Quality Assessment for wheat-sensory Tests for Noodles. Ministry of Agriculry and Fisheries, National foods Research Institute, Japan.

Bhattacharya M, Zee SY, Corke H (1999) Physicochemical properties related to quality of rice noodles. Cereal Chem. 76(6):861-867.

Bourne MC (1978) Texture profile analysis. Food Technol. 32(7):62-72.

Chakraborty M, Hareland GA, Manthey FA, Berglund LR (2003) Evaluating quality of yellow alkaline noodles made from mechanically abraded sprouted wheat. J. Sci. Food Agr. 83(5):487-495.

Del Nobile MA, Baianoa A, Contea A, Mocci G (2005) Influence of protein content on spaghetti cooking quality. J. Cereal Sci. 41(3):347-356.

Dick JW, Youngs VL (1988) Evaluation of durum wheat, semolina, and pasta in the United States. Pages 237-248 in: Durum Colour Chemistry and Technology. G. Fabriani and C. Lintas, eds. Am. Assoc. Cereal Chem., St. Paul, MN.

Doores S (1993) Organic acids. In: Davidson PM and Branen AL (ed) Antimicrobials in foods. New York, U.S.A. pp. 95-136.

Forsythe SJ (2000) The Microbiology of Safe Food. Blackwell Science, Oxford. pp. 412, ISBN 0-632-05487-5.

Fu X, Huang B, Feng F (2008) Shelf life of fresh noodles as affected by the food grade monolaurin microemulsion system. J. Food Proc. Eng. 31(5):619-627.

Ghaffars S, Abdulamir AS, Baker FA, Karim R, Saari N (2009) Microbial growth, sensory characteristics and pH as potential spoilage indicators of Chinese yellow wet noodles from commercial processing plants. Am. J. Appl. Sci. 6(6):1059-1066.

Hou G, Kruk M (1998) Asian noodle technology. Asian Noodle Technical Bull. 20(12):1-10.

Hou G (2001) Oriental noodles. Adv. Food Nutr. Res. 43:143-193.

Howard JG, Reiter HJ, Fritsch D, Scheren B, Lafleur TJ (1994) Acid Stabilized Pasta. United State Patent: US 5332-587.

Hu XZ, Wei YM, Wang C, Kovacs MIP (2007) Quantitative assessment of protein fractions of Chinese wheat flours and their contribution to white salted noodle quality. Food Res. Int. 40(1):1-6.

Jianming C (1998) Preservation of fresh noodles by irradiation. Radiat. Phys. Chem. 52(1-6):35-38.

Karim R (1989). Textural Studies on Chinese Wet Noodles (Hokkien Styles Noodles). Thesis, University Putra Malaysia.

Keiithiro M, Fusaki K, Akio F, Futoshi H (2001) Processes for the production of noodles by machines. United States US. Patent 6:197-360.

Lee L, Baik BK, Czuchajowska Z (1998) Garbanzo bean flour usage in cantonese noodles. J. Food Sci. 63(3):552-558.

Lee DS, Paik HD, Im GH and Yeo IH (2001) Shelf life extension of Korean fresh pasta by modified atmosphere packaging. J. Food Sci. Nutr. 6:240-243.

Li M, Zhu K, Guo X, Peng W, Zhou H (2011) Effect of water activity and irradiation on the shelf- life of fresh noodles. Innov. Food Sci. Emerg. 12:526-530.

Li M, Zhu K, Guo X, Brijs, K, Hui-Ming Zhou, H (2014) Natural Additives in Wheat-based pasta and nNoodle products: opportunities for enhanced nutritional and functional properties. Compr. Rev. Food Sci. Food Saf. 13(4):347-357.

Li M, Zhu K, SunQ, Amza T, Guo X and Zhou H (2016) Quality characteristics, structural changes, and storage stability of semi-dried noodles induced by moderate dehydration: Understanding the quality changes in semi-dried noodles. Food Chem. 194:797-804.

Malyniak NR, Meagher JF (1990) Food preservation process utilizing a blend of acidulants. United States Patent: US 4931297 A.

McGuire M, DiGiacomo R, Palmer M, Ligget L (1989) Method for preparing and perserving fresh pasta. United State Patent: US 4:876-104.

Moss H J, Gore PJ, Murray IC (1987) The influence of ingredient and process variables on the quality and microstructure of Hokkien, Cantonese and instance noodles. Food Microstruct. 6(1):63-74.

Ng CC, Wang CY, Wang YP, Tzeng WS, Shyu YT (2011) Lactic acid bacterial fermentation on the production of functional antioxidant herbal *Anoectochilus formosanus Hayata*. J. Biosci. Bioeng. 111(3):289-293.

Oh NH, Seib PA, Deyoc CW, and Ward, AB (1985) Noodles. II. The surface firmness of cooked noodles from soft and hard wheat flours. Cereal Chem. 62(2):431-436.

O'Rourke J, Tunstall JA, Howe SC, Moberg LJ (2003) Method for thermal processing and acidification of pasta product. United State Patent: US 6,576,279.

Piddock LG (1990) Techniques used for the determination of antimicrobial resistance and sensitivity in bacteria. J. Appl. Bacteriol. 68(4):307-318.

Ray B (2001) Fundamental food microbiology. CRC Press. pp. 97-228.

Ricke SC (2003) Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poult. Sci. 82(4):632-629.

Saito K, Nishio Z, Takata K, Kuwabara T, Yokota A, Yamauchi H (2003) Bacterial adjunct to improve the quality of alkaline noodles. Food Sci. Technol. Res. 9(1):40-44.

Shelke K, Dick JW, Holm YF, Loo KS (1990) Chinese wet noodle formulation: a response surface methodology study. Cereal Chem. 67(4):338-342.

Shiau SY (1996) Effects of additives and temperature on the physical and chemical properties of doughs and extruded noodles. Dissertation of National Taiwan University.

Shiau SY and Yeh AI (2001) Effects of alkali and acid on dough rheological properties and characteristics of extruded noodles. J. Cereal Sci. 33(1):27-37.

Tanaka K, Furukawa K, Matsumoto H (1967) The effect of acid and salt on the farinogram and extensigram of dough. Cereal Chem. 44(6):675-680.

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Potential of certain cultivars and resistance inducers to control gray mould (*Botrytis cinerea*) of pepper (*Capsicum annuum* L.)

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Four different highly economic value pepper cultivars Trezaa, Emberu, Mazurka and Taranto usually grown in Egypt were used to evaluate the efficacy of certain resistance inducers to enhance resistance of pepper fruits against gray mould fungus. Pathogenicity tests with *Botrytis cinerea* (BC-3 isolate) revealed that cv. Mazurka (Red Colour) was most tolerant and exhibited 40.15% rot severity, while cv. Emberu (Yellow Colour) was highly susceptible and showed 79.12% rot severity. The other two pepper cultivars, that is, cv. Trezaa and Taranto showed severity of 50.23 and 52.75%, respectively. Treatment of pepper fruits with resistance inducers salicylic acid (SA); abscisic acid, methyl jasmonate and calcium chloride significantly decreased gray mould development under laboratory conditions. Calcium chloride was the most effective on all pepper cvs. mentioned earlier and inhibited diameter of rotting area with overmean of 40.6%. This was followed by SA as 34.01%. Abscisic acid and methyl jasmonate, however, showed lower mean inhibition of 16.8 and 18.8%, respectively. Meantime, treatment of pepper fruits with such resistance inducers and calcium chloride increased activity of the defense related enzymes, that is, polyphenol oxidase (PPO), peroxidase (POD), phenylalanine ammonia lyase (PAL) as well as the total phenols where SA and calcium chloride were the most effective. This could explain potentiality of such compounds to enhance pepper resistance to control *B. cinerea* of the gray mould of pepper.

Key words: Pepper, gray mould, *Botrytis cinerea*, resistance inducers, calcium chloride, defence enzymes, phenolic content.

INTRODUCTION

Sweet pepper (*Capsicum annuum L.*) is one of most popular and favorite vegetable crops cultivated in Egypt for local market and exportation. Such high cash crop has occupied an important rank in the Egyptian and world

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agriculture due to its high profit and nutritional value (El-Hifny and El-Sayed, 2011). Pepper is grown extensively both in the field and in the greenhouse. Heavy losses in yield and quality occur in pepper, because of a number of diseases (Zitter, 2011). Gray mould fungus, *Botrytis cinerea* is an ubiquitous fungus distributed worldwide and reported to be a pathogen of plants in more than 200 genera, including pepper, which grown in enclosures that maintain high relative humidity and causes postharvest decay (Pernezny et al., 2003). No pepper cultivars were recorded so far to be resistant to gray mould, while, little number of cultivars were reported to be tolerant to different degree in different parts of the world (Elad and Shtienherg, 1995; Elad et al., 2004). Control of this disease is especially important during storage, because it develops at low temperature and spreads quickly among fruits and vegetables (Karabulut et al., 2004). Losses can be ameliorated by fungicide treatments (Rosslenbroich and Stuebler, 2000). However, public concerns about the negative impact of the synthetic fungicide residues on human health and environment have prompted the search for safer alternatives (Houeto et al., 1995; Meng et al., 2008). Resistance inducers such salicylic acid (SA), jasmonates, and abscisic acid as well as the inorganic resistance inducer elicitor calcium chloride have been reported to play an important role in systemic signaling systems triggering expression of various defense-responsive genes (Leon and Daryl, 2004; Denancé et al., 2013; Yang et al., 2013; Alazem et al., 2014; Kim and Hwang, 2014). During defense communication, SA plays a role in both local and systemic resistance reactions and it combats invading pathogens due to its natural antimicrobial properties (Murphy and Carr, 2002).

Recently, it has been observed that SA treatment could be used to reduce deterioration and chilling injury symptoms in some fruit (Sayyari et al., 2009). Both pre- and post-harvest SA treatments have been reported as being effective in fruit quality maintenance and storage life extension of strawberry (Babalare et al., 2007). Preharvest application of SA has induced resistance against pathogens in pear (Jiankang et al., 2006) and decreased disease development in cherry (Yao and Tian, 2005). Application of exogenous SA at non-toxic concentrations to fruit has been shown to delay the ripening and softening of banana (Srivastava and Dwivedi, 2000), reduce lipid peroxidation of navel orange (Huang et al., 2008), and increase host resistance to postharvest diseases of sweet cherry (Qin et al., 2003). A few studies have reported the effects of SA on chilling injury, showing that SA and methyl salicylate (MeSA) treatments increase resistance to postharvest chilling injury in horticultural crops, including tomato (Ding et al., 2001), peach (Wang et al ., 2006) and (Cao et al., 2010), pomegranate (Sayyari et al., 2009), and pineapple (Lu et al., 2010). On the other hand, SA application either preharvest (Yao and Tian, 2005) or postharvest reduced fungal decay in sweet cherry through induction of the defense resistance system (Chan and Tian, 2006) and stimulation of antioxidant enzymes (Xu and Tian, 2008).

Jasmonic acid signaling is crucial in regulating defense responses against necrotrophic pathogens and it also plays an important role through the induced systemic resistance (Pieterse et al., 2009). Abscisic acid (ABA) is called the hormone controlling plant response to fungal, bacterial, and viral stress (Mohr and Cahill, 2003, 2007). The role of abscisic acid (ABA) in plant defense is not well understood and even controversial. ABA mostly studied as a global regulator of abiotic stress adaptation, and it has recently emerged as a key determinant in the outcome of plant-pathogen interactions (Thaler and Bostock, 2004; Achuo et al., 2006; Asselbergh et al., 2007). Calcium was found to reduce plant susceptibility to fungal infection in some pathosystems (Vale et al., 2000). Calcium plays an important role in the defense of plants against *Sclerotinia sclerotiorum* since it is essential in the structure of the middle lamellae of plant cells and in maintaining selectivity of cell plasma lemma (Maxwell and Lumsden, 1970; Godoy et al., 1990; Zhou and Boland, 1999). Moreover, it has been frequently reported that $CaCl_2$ and SA extended shelf life of several vegetables and fruits (Dimitrios and Pavlina, 2005; Hernandez-Munoz et al., 2006; Ramezanian and Rahemi, 2007; Arhtar et al., 2010; Chen et al., 2011; Rao et al., 2011). Plant enzymes and phenols were found to be involved in defense reactions against plant pathogens, this include oxidative enzymes such polyphenol oxidase and peroxidase (Thilagavathi et al., 2007) which involved in oxidization of phenols and lignification of plant cells during the microbial invasion (Kolattukudy et al., 1992; Chittoor et al., 1999). Other enzymes such phenylalanine ammonia-lyase were involved in phytoalexin or phenolic compound biosynthesis (Yedidia et al., 2000; Anesini et al., 2008). The present study therefore was conducted to: (a) reveal the causal fungus responsible for gray mould and to reveal amount of variation existed in its population in four major regions for pepper cultivation in EL-Beheira governorate, (b) to study potential of four widely grown pepper cultivars to control the gray mould fungus and, (c) to evaluate the efficacy of certain resistance inducers as alternative for fungicides to control post-harvest gray mould of pepper.

MATERIALS AND METHODS

Sampling, isolation and identification of the causal fungus

Samples of diseased pepper (*C. annuum* L.) fruits showing symptoms of gray mould were collected from greenhouses in four major regions for pepper cultivation in El-Beheira governorate, that is, Hosh Issa, EL-Dillingate, EL-Nubaria and Wadi El-Natrun during the 2012 to 2013 growing seasons. Samples were separately kept in polyethylene bags and directly transferred to laboratory for

isolation of the causal fungus. Diseased fruits were washed in running tap-water, and then surface disinfested with 70% ethanol. A small portion of symptomatic tissues was plated on potato dextrose agar (PDA) and incubated at 22 to 25°C for 3 to 6 days. A pure culture was established using hypal tip and single spore isolation techniques. Cultures were maintained on PDA and stored for short periods in refrigerator. The isolates obtained were identified based on their morphological and cultural characters according to Raposo et al. (1995).

Pathogenicity tests

Pathogenicity tests of the recovered isolates were conducted on the susceptible cv. Emberu (yellow) of pepper. Pepper fruits were washed with 1% sodium hypochlorite for 1 min, rinsed with sterilized distilled water and air-dried at room temperature (25°C) for 30 min and then placed in sterile plastic containers containing moist cotton. Fruits were wounded at one side to a depth of 2 mm and inoculated with a 0.5 cm disc taken from the edge of PDA culture of the causal fungus or free disc of PDA as a control. Five replicates for each treatment were conducted. Diameters of rotting area were measured seven days after inoculation and incubated at 22 to 25°C, and taken as a criterion for virulence of the recovered isolates (Ozdemir and Floros, 2004).

Susceptibility of different pepper cultivars to gray mould fungus

Under laboratory conditions, healthy pepper fruits of four widely grown cultivars, that is, Taranto and Emberu (yellow fruits) and Mazurka and Trezaa red fruits were used to study their susceptibility to gray mould fungus isolates. A highly virulent isolate, according pathogenicity test, of the gray mould fungus was used in the study. Pepper fruits were inoculated as previously mentioned in the pathogenicity test. Five replicates of each cultivar were conducted. The diameters of rotting area were estimated in centimeters, seven days after inoculation and incubation at 22 to 25°C.

Effect of certain resistance inducers on gray mould of pepper

Four pepper cultivars previously tested for their susceptibility were used to evaluate the efficacy of certain resistance inducers to enhance resistance of pepper fruits against gray mould fungus. A highly virulent *B. cinerea* isolate was used in the study. Pepper plants of the four cultivars were planted in a commercial greenhouse at EL-Dillingate region in El-Beheira governorate during 2014 growing season. The cultural practices were carried out according to the recommended practices followed in this area. Plants were never treated with any fungicide. The plants were sprayed with solutions of SA (2-hydroxybenzoic acid; C7 H_6O_3) at 1 mM, methyl jasmonate (MeJA) with concentration of 100 µM and abscisic acid (ABA) with concentration of 0.1 mM as organic compounds and calcium chloride as non-organic resistance inducers compound at 1.5 g/L, after 45 days of cultivation. Treatments were repeated weekly for four weeks until fruit harvest. Plants sprayed by distilled water were used as a control. All chemicals were sigma products. At harvest, fruits of pepper of the same four cultivars were collected in polyethylene bags and transferred to laboratory, washed in running tap-water, then disinfested with sodium hypochlorite 1% for 1 h and finally rinsed in sterile water before being allowed to dry. Fruits were then sprayed with solutions of the resistance inducers at the same concentration used in the greenhouse and kept to dry at room temperature for 30 min. Pepper fruits were then inoculated with a highly virulent gray mould isolate as mentioned in the pathogenicity test. Five replicates for each treatment were conducted. Diameters of rotting area were estimated in centimeters seven days after inoculation and incubation at 22 to 25°C.

Determination of the defense related enzyme activity and total phenols

The four pepper cultivars of different degrees of susceptibility to gray mould, that is, Taranto, Mazurka, Emberu and Trezaa were used to reveal the differences in defense enzyme activities and total phenols between pepper cultivars and after treatment with resistance inducers. This included polyphenol oxidase (PPO), peroxidase (POD), phenylalanine ammonia lyase (PAL) and total phenolic content. Also pepper fruits were sprayed with the resistance inducers compounds and inoculated with a highly virulent isolate of gray mould fungus as previously mentioned. Fruits sprayed with water were used as a control. Enzyme activities and total phnoles were evaluated in pepper after 0, 12, 24, 72 and 168 h after inoculation. Each treatment was represented by five replicates.

Estimation of polyphenol oxidase (PPO) activity

One gram of the pepper fruits was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) in a pre-chilled pestle and mortar. The homogenate was centrifuged at 12,000 g for 15 min at 4°C and the supernatant served as enzyme source. Polyphenol oxidase activity was determined as the procedure given by Mayer et al. (1965) with some modification. The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M catechol was added. The reaction mixture was incubated at room temperature and the absorbance was set to zero at 398 nm. The changes in absorbance were recorded at 30 s interval for 2 min and the activity was expressed as change in absorbance min^{-1} g^{-1} of fresh tissue.

Estimation of peroxidase (POD) activity

The peroxidase activity was assayed as described by Chen et al. (2000). Extraction was carried out by homogenizing 1 g of the pepper fruit in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) using pre chilled pestle and mortar (4°C). The homogenate was centrifuged at 12,000 g for 15 min at 4°C. The supernatant served as enzyme source and the reaction mixture consisted of 1.5 ml of 0.05 M Pyrogallol, 0.5 ml of enzyme extract, and 0.5 ml of 1% H_2O_2. The reaction mixture was incubated at 28±2°C. At the start of enzyme reaction, the absorbance of the mixture was set to zero at 460 nm in the spectrophotometer and the change in the absorbance was recorded at 20 s interval for 3 min. The peroxidase activity was expressed as change in the absorbance of the reaction mixture min^{-1} g^{-1} of fresh tissue.

Estimation of phenylalanine ammonia lyase (PAL) activity

One gram of the pepper fruits was homogenized in 3 ml of ice-cold 0.1 M sodium borate buffer, pH 7.0 containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinylpyrrolidone (PVP). The extract was filtered through cheese cloth and the filtrate was centrifuged at 15,000 g for 15 min at 4°C. The supernatant was

Table 1. Frequency of virulence of *Botrytis cinerea* isolates recovered from pepper fruits showed gray mould symptoms collected during 2012-2013 growing seasons from four regions in EL-Beheira governorate.

Region	Total number of isolates	Frequency of virulence			
		Low	Moderate	High	Very high
El-Delengat	8	1	3	3	1
Wadi El -Natrun	6	1	2	3	-
EL-Nubaria	5	1	1	3	-
Haush Essa	5	-	3	2	-
Total	24	3	9	11	1
Ratting (%)	-	12.5%	37.5%	45.83%	4.17%

Low virulence= gray mould rot covered less than 25% of the fruit surface, moderate= rot covered 25- 50% of the fruit surface, High = rot covered > 50-75% and Very high= rot covered > 75% of the fruit surface.

used as enzyme source. Phenylalanine ammonia lyase activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (Dickerson et al., 1984). Sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 M^{-1} cm^{-1}. Enzyme activity was expressed as synthesis of trans-cinnamic acid (in µmol quantities) min^{-1} g^{-1} fresh weight.

Estimation of total phenols

Total phenolic content of pepper fruit was estimated by Folin Ciocateau method (Zieslin and Ben Zaken, 1993) with some modification. One gram of sample was homogenized in 10 ml of 80% methanol and agitated for 15 min at 70°C, then 1 ml of methanolic extract was added to 5 ml of distilled water and 250 µl of Folin-Ciocateau reagent and incubated at 25°C; after 3 min, 1 ml of the saturated solution of sodium carbonate and 1 ml of distilled water were added and the reaction mixtures were incubated further for 1 h at 25°C. The absorption of the developed blue color was measured using spectrophotometer at 725 nm. The total soluble phenol content was calculated according to a standard curve obtained from a Folin-Ciocateau reagent with a phenol solution and expressed as catechol equivalent g of fresh tissue.

Effect of storage temperature on development of gray mould in pepper fruits treated with SA and calcium chloride

Fruits of peppers cv. Mazurka and cv. Emberu were washed in running tap-water, disinfested with sodium hypochlorite (1%) for 5 min and finally rinsed in sterile water before being allowed to dry. Then, peppers were immersed in aqueous solutions of calcium chloride at 1.5 g/L, and SA at 1 mM for 60 min, and were left to dry at room temperature for one day before being inoculated (Hajhamed et al., 2007). Fruits immersed in water were used as negative control. Inoculated fruits with (*B. cinerea*, *BC-3)* isolate were used as positive control. After three, seven and eleven days of incubation at 8, 12 and 25°C, the diameters of rotting area were estimated in centimeters. Each treatment was represented by five replicates.

Virulence and disease assessments

Virulence of the recovered isolates and severity of the developed gray mould on pepper fruits in the different experiment were conducted according to Balogun et al. (2005) as follows:

Low virulence= gray mould covered ≤25% of fruit surface;
Moderat virulence= gray mould covered >25-50% of fruit surface;
High virulence= gray mould covered >50-75% of fruit surface;
Very high virulence= gray mould covered >75% of fruit surface

$$\text{Severity (\%)} = \frac{\text{Mean diameter of rotting aera}}{\text{Mean diameter of fruit surface}} \text{x100}$$

Statistical analysis

The obtained data were statistically analyzed according to Gomez and Gomez (1984) using the SAS program version 9.2 (SAS Inc., 2009). Means were compared using the least significant difference (LSD) test at 0.05 level of probability.

RESULTS

Fungi associated with gray mould of pepper

Twenty four *B. cinerea* isolates were recovered from diseased pepper fruits showed gray mould symptoms collected from four major regions for pepper cultivation in El-Behera governorate, that is, Wadi El-Natrun, EL-Nubaria, El Delengat and Haush Essa during the 2012 to 2013 growing season (Table 1). Eight of the *B. cineria* isolates were recovered from El Delengat pepper samples, while six isolates were isolated from Wadi El Natrun and also five isolates were isolated from each of EL-Nubaria and Haush Essa regions (Table 1).

Pathogenicity tests

Pathogenicity tests of the twenty four *B. cinerea* recovered in the survey were conducted on the susceptible pepper cv. Emberu (yellow pepper). The obtained data presented in Table 1 showed that all the

Table 2. Susceptibility of four pepper cultivars to the artificial infection with *Botrytis cinerea* (*BC-3* isolate).

Cultivars	Fruit Colour	Diameter of rotting area (cm)*	Rot severity (%)
Emberu	Yellow	5.250[a]	79.12
Trezaa	Red	3.500[b]	52.75
Taranto	Yellow	3.333[b]	50.23
Mazurka	Red	2.667[c]	40.15

*Data are average of five replicates.

tested isolates were virulent to different degrees on the tested peppers cultivar. However, only one isolate (that is, 4.17% of the total) was recognized as very highly virulent as incited gray mould on pepper more than 75% of the fruit surface, seven days after inoculation. Meantime, eleven isolates out of the 24 analyzed (that is, 45.83% of the total) were highly virulent and incited gray mould of > 50%-75% on the fruit surface. Also, nine isolates (37.5%) were moderate and incited gray mould of 25 to 50% on the fruit surface, while three isolates (that is, 12.5% of the total) showed low virulence as gray mould covered less than 25% of the fruit surface (Table 1).

Susceptibility of pepper cultivars to *B. cinerea*

Data in Table 2 shows that all tested pepper cultivars, that is, Emberu, Trezaa, Mazurka and Taranto were susceptible to the infection with *B. cinerea* (*BC-3* isolate). The cv. Emberu (yellow) was highly susceptible as gray mould developed with artificial inoculation covered 79.12% of the fruit surface, seven days after inculation. However, cv. Mazurka (Red) showed the least susceptibility (most tolerant) as gray mould covered only 40.15% of the fruits surface. Meanwhile, cv. Trezaa (Red) and cv. Taranto (yellow) cultivars exhibited intermediate susceptibility as gray mould developed with artificial inoculation covered 52.75 and 50.23% of the fruits surface, respectively (Table 2). Values followed by different letters are significantly different at p= 0.05 of probability.

$$\text{Rot severity (\%)} = \frac{\text{Mean diameter of rotting aera}}{\text{Mean diameter of fruit surface}} x100$$

Enzyme activity and total phenols in relation to susceptibility of pepper cultivars to *B. cinerea*

The defense related enzyme activity and the total phenols contents were determined in pepper fruits of the four tested cultivars for their susceptibility to *B. cinerea* (*BC-3* isolate). Data illustrated in Figure 1A and B showed that the enzyme activities of PPO, POD, PAL as well as total phenols constantly increased in pepper fruits of the four cvs. after inoculation with *B. cinerea* (*BC-3* isolate) compared with non -inoculated control. Meantime, it was obvious that the most tolerant cv. Mazurka showed the highest activity for all tested enzymes and total phenols in both the uninoculated and inoculated pepper cvs. The highest peak is as early as 24 h after inoculation. On the contrary, the most susceptible cultivar, that is, cv. Emberu showed the lowest enzymes activities and total phenols with highest peack as late as 72 h after inoculation. The two other cultivars, that is, cv. Taranto and cv. Trezaa of the intermediate susceptibility showed intermediate enzyme activities and total phenols values.

Effect of certain resistance inducers on gray mould of pepper

SA, methyl jasmonate (MeJA), abscisic acid (ABA) and calcium chloride(Ca Cl_2), were tested for their potentiality to enhance resistance of pepper fruits of different cultivars, that is, Trezaa, Emberu ,Mazurka and Taranto against the highly virulent *B. cinerea* (*BC-3* isolate).
Data in Table 3 showed that gray mould developed on the fruits mostly significantly decreased with the treatment of the tested resistance inducers on the four pepper cvs compared with the untreated inoculated control. SA and calcium chloride were the most effective on all tested cultivars and significantly decreased mean developed rot by 34.01 and 40.6%, respectively. However, abscisic acid and methyl jasmonate inhibited the mean gray mould developed by 16.8 and 18.8%, respectively.

Effect of resistance inducer on defense related enzyme activity and total phenols

Pepper cv. Emberu was used to determine the effect of resistance inducers and calcium chloride on the defense related enzyme activity and total phenols in treated fruits and then inoculated with *B. cinerea (BC*-3). It was evident

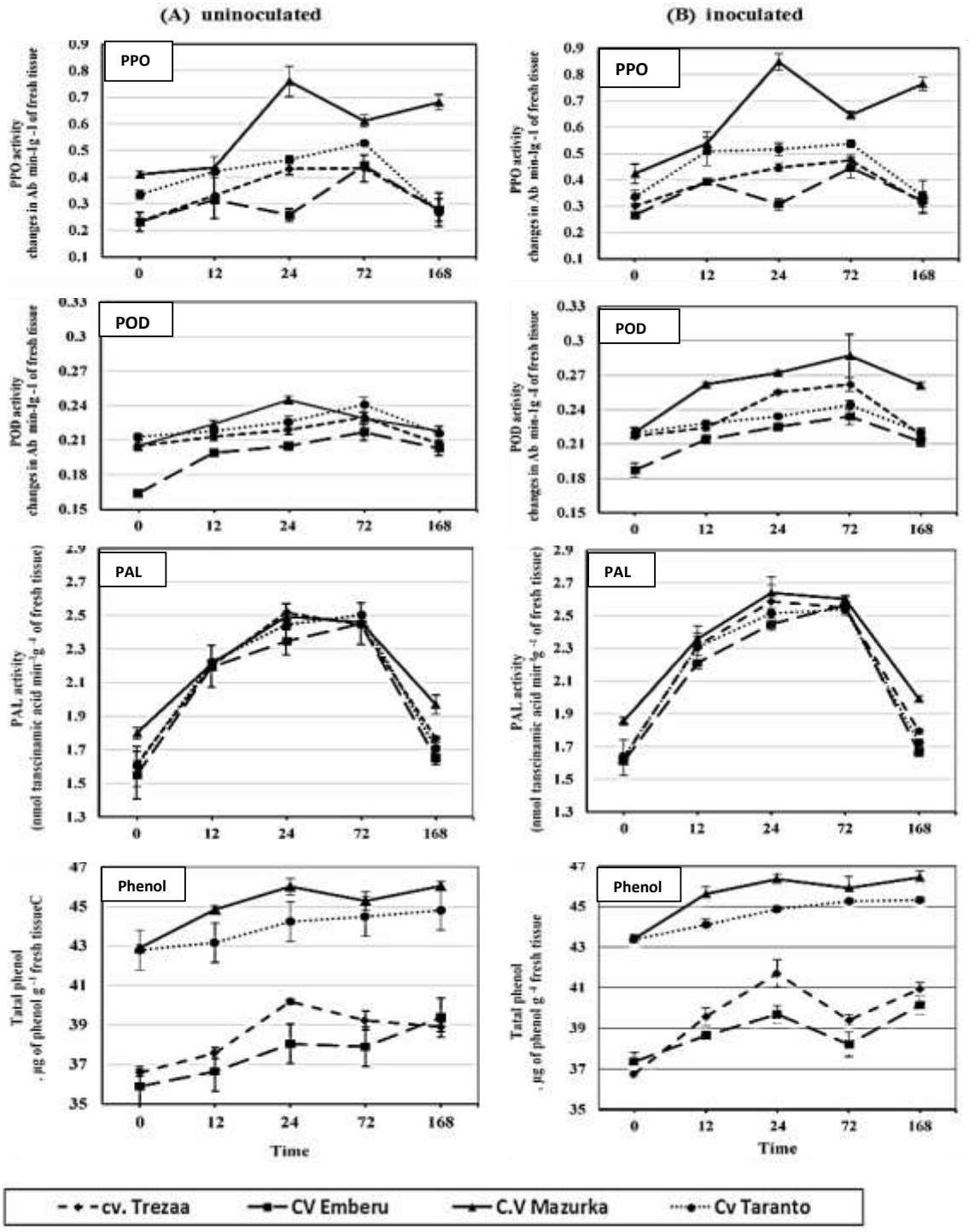

Figure 1. Enzyme activity of polyphenol oxidase (PPO), peroxidase (POD), and phenylalanine ammonia lyase (PAL) and the total phenol content in pepper fruits of four pepper cultivars, uninoculated (A) and inoculated (B) with *B. cinerea* (*BC-3* isolate).

(Figure 2) that all treatments obviously increased enzyme activity of the tested defence related enzymes as well as the total phenols content compared with the inoculated untreated control. This was steady over the 168 h period

Table 3. Effect of treatment with certain resistance inducers and calcium chloride on diameter of gray mould developed on pepper fruits of four tested cultivars treated and then inoculated with *B. cinerea (BC-3)* under laboratory conditions, seven days after inoculation.

Treatment	Pepper cultivars									
	Cv. Emberu		Cv. Trezaa		Cv. Taranto		Cv. Mazurka		Mean	
	Diameter (cm) *	% Inhibition	Diameter (cm) *	% Inhibition	Diameter (cm) *	% Inhibition	Diameter (cm) *	% Inhibition	Diameter (cm) *	% Inhibition
Jasmonate (MeJA)	4.250^{bc}	23.32	3.103^{ab}	16.36	2.977^{a}	7.55	2.000^{ab}	27.98	3.082^{b}	18.80
Abscisic acid (ABA)	4.330^{b}	21.88	3.12^{a}	15.71	2.877^{a}	10.65	2.250^{ab}	18.98	3.146^{b}	16.80
Salicylic acid (SA)	3.667^{cd}	34.60	2.410^{bc}	35.04	2.427^{b}	24.63	1.617^{b}	41.77	2.529^{c}	34.01
Calcium chloride (Ca Cl_2)	3.627^{d}	34.57	1.980^{b}	46.63	2.330^{b}	38.20	1.583^{b}	42.99	2.379^{c}	40.60
Infected -un treated (control)	5.543^{a}	-	3.710^{a}	-	3.220^{a}	-	2.777^{a}	-	3.811^{a}	-
Mean	4.283^{a}	-	2.864^{b}	-	2.764^{b}	-	2.045^{c}	-		-

*Values are means of five replications. Values for each parameter followed by different letters are significantly different at p=0.05 of probability.

of the investigation. The highest peak was recognized 24 h after inoculation for PAL and total phenols, while it was relatively later at 72 h for PPO and POD. Meantime, SA was the most effective to induce activity of PPO and POD, while $CaCl_2$ was most effective to induce PAL activity and total phenols.

Effect of storage temperature on development of gray mould

Data illustrated in Figure 3 showed that a strong positive correlation (r = 0.6 to 0.9) was revealed between storage degrees of temperature and the developed diameter of gray mould on both tested pepper cultivrs. Diameter of the rotting area decreased with decreasing storage temperature and vice versa where 8°C exhibited the least diameter of gray mould. Meanwhile, treatment of pepper fruits with calcium chloride and SA before storage led to a further decrease in the developed gray mould during storage and calcium chloride (1.5 g/L) exhibited the highest effect (Table 4). On the other hand, data tabulated in Table 4 also showed that, calcium chloride effectively delayed time of appearance of the first signs of gray mould (that is, increased shelf life) on both tested cultivars as there were no gray mould symptoms up to seven days after inoculation with *B. cinerea* (*BC-3* isolate).

DISCUSSION

Sweet pepper (*C. annum L*.) is one of the most important high value crop grown extensively throughout the world, especially in the temperate region. Gray mould of pepper caused by the fungus *Botrytis cinerea* is the most important disease of pepper worldwide in the field and under post-harvest conditions (Vagelas et al., 2009). Twenty four isolates of *B. cinerea* were isolated from diseased pepper fruits showed gray mould symptoms collected from four major regions for pepper cultivation in El-Behera governorate, Egypt. All fungal isolates were virulent to the susceptible cv. Emberu to different degrees. On the other hand, all pepper cultivars, that is, Taranto, Emberu, Mazurka and Trezaa were susceptible to the gray mould fungus *B. cinerea* (*BC-3* isolate). Searching for resistant pepper cultivars against post-harvest disease is restricted (Kiran et al., 2006). Plant pathologists worldwide are more concerned about dealing with threats of using synthetic fungicides (Okigbo, 2009). Finding safe alternative methods of pathogen control is obligatory (Ijato et al., 2011; Nsabiyera et al., 2012). One of the most well-known methods of plant prevention to plant pathogenic fungi is chemical control by deploying different groups of fungicides (Yaqub and Shahzad, 2006). Chemical control can generate resistance of fungi to fungicides and can cause environment pollution with direct effect of human health (Zhang et al., 2007; Damalas and Eleftherohorinos, 2011). In recent years, the trend of using different chemicals in plant production has been minimized. Thus, there is need for an efficient,

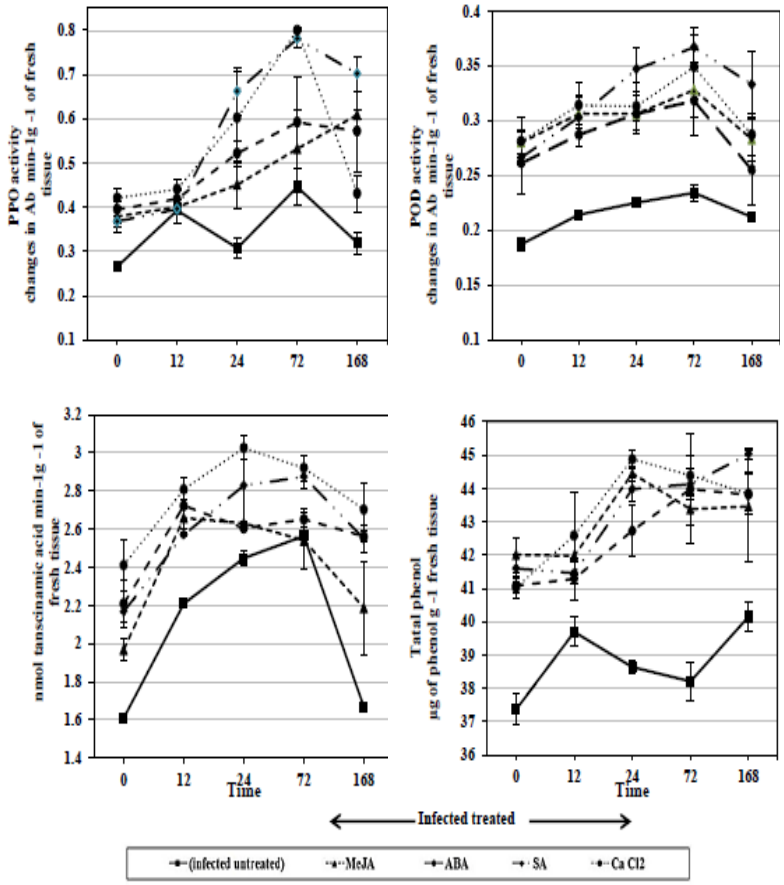

Figure 2. Enzymes activity in pepper fruits of cv. Emberu treated with jasmonate (MeJA), Abscisic acid (ABA), salicylic acid (SA) and calcium chloride (Ca Cl_2) and inoculation with *B. cinerea* (*BC-3)* isolate.

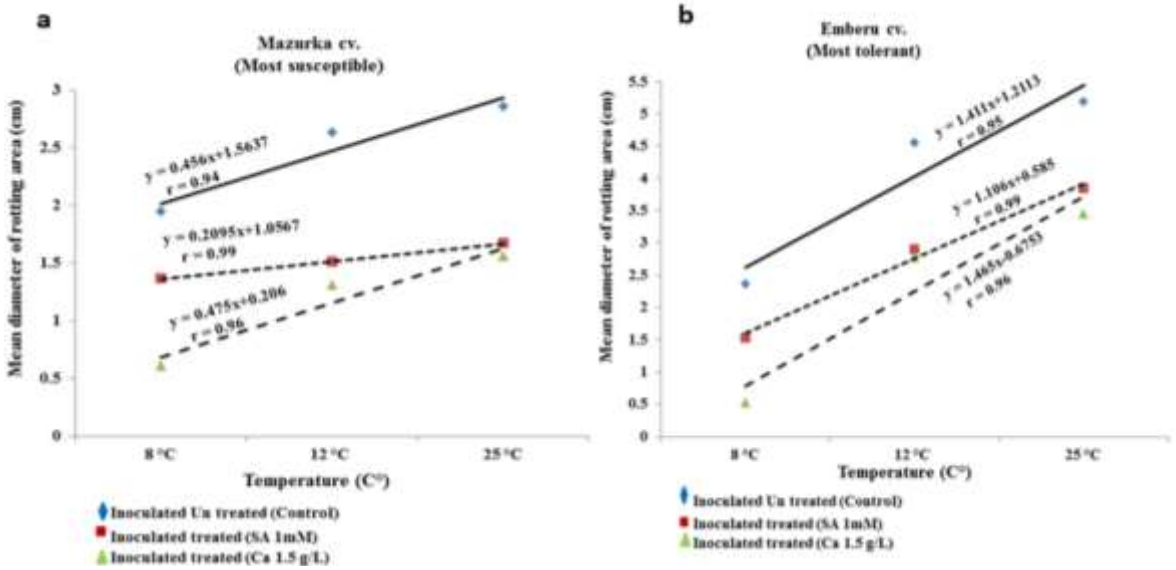

Figure 3. Correlation between storage temperature and development of gray mould on pepper cv. Mazurka (a) and cv. Emberu (b) treated with salicylic acid and calcium chloride and inoculated with *B. cinerea* (*BC-3*).

Table 4. Effect of storage at 8°C on development of gray mould on pepper fruits treated with salicylic acid and calcium chloride and inoculated with *B. cinerea* (*BC-3*).

Treatment	Diameter of rotting area (cm)*							
	Cv. Mazurka				Cv. Emberu			
	3 days	7 days	11 days	Mean	3 days	7 days	11 days	Mean
Fungus (*BC-3*) Control	0.333	2.250	3.250	1.944[a]	0.417	2.667	4.000	2.361[a]
SA (1 mM) + Fungus	0.000	1.667	2.083	1.250[b]	0.333	1.9167	2.667	1.638[b]
Ca Cl_2 (1.5 g/L) + Fungus	0.000	0.000	1.816	0.606[c]	0.000	0.000	1.583	0.528[c]
Mean	0.139[c]	1.306[b]	2.383[a]	-	0.139[c]	1.528[b]	2.750[a]	-

*Values are means of three replications.

environmental-safe method to control plant diseases.

SA, methyl jasmonate (MeJA) and calcium chloride is a natural compound that plays a central role in disease resistance (Delaney et al., 1994; El-Ghouth et al., 2000; Darras et al., 2005; Yao and Tan, 2005; Jin et al., 2009). SA, methyl jasmonate (MeJA), Abscisic acid (ABA) and calcium chloride were considered as systemic acquired resistance (SAR) inducers (Mengiste et al., 2010) were tested in the present study for their potential to enhance resistance of pepper cvs. against gray mould fungus *B. cinerea.* Calcium chloride was the most effective and decreased diameter of rotting area by 46.63, 42.99, 38.20 and 34.57% on the tested pepper cvs. Trezaa, Mazurka, Taranto and Emberu, respectively, with over mean of 40.6%. This was followed by SA which inhibited diameter of rotting area by 35.04, 41.77, 24.63 and 34.60% on the mentioned cvs. respectively, with over mean of 34.01%. Abscisic acid and methyl jasmonate however showed lower mean inhibition of 16.8 and 18.8%, respectively. The obtained results could be explained in view that application of $CaCl_2$ at postharvest stage successfully inhibited spore germination and thus provided a good control for *B. cinerea* on apple (El-Gali, 2008). Also fruits containing high concentration of Ca were found to be less susceptible to the fungal infection (Naradisorn, 2013). On the other hand, SA was involved in reducing the damage caused by various pathogens such bacteria, fungi and viruses (Raskin, 1992). It is considered an important factor in systemic acquired resistance (SAR) against many pathogens (Nie, 2006). Meanwhile, jasmonic acid signaling molecules in plants make the plant responsive to various biotic and abiotic stresses (Wasternack and Hanse, 2013; Zhou et al., 2013). Jasmonic acid was also involved in fruit ripening and plant response to injury as well as resistance to insects and pathogens (Meng et al., 2009; Jin et al., 2009). However, the role of abscisic acid (ABA) in plant defense is not well understood and even controversial (Mohr and Cahill, 2003, 2007). Exogenous application of ABA increased the susceptibility of various plant species to bacterial and fungal pathogens (Thaler et al., 2004; Achuo et al., 2006; Asselbergh et al., 2007). Disruption of ABA biosynthesis was shown to confer resistance to the necrotroph *B. cinerea* (Audenaert et al., 2002).

Plant enzymes were shown to be involved in defense reactions against plant pathogens (Lebda et al., 2001; Elad et al., 2004). This included oxidative enzymes such polyphenol oxidase (PPO), peroxidase (POD) and phenylalanine ammonia lyase (PAL) (Thilagavathi et al., 2007). Also plant Phenols as secondary metabolites were recorded to initiate a number of defensive reactions against plant pathogens (Lattanzio et al., 2006). In the present study, the highest levels in enzyme activity of PPO, POD, and PAL as well as the total phenols were revealed in the most tolerant pepper cv. Mazurka while the lowest levels were recorded in the most susceptible cv. Emberu of pepper. Our findings were in agreement with earlier studies of Chittoor et al. (1997) and Li and Steffens (2002). The present study however, showed that treatment of pepper fruits with such resistance inducers and calcium chlorides increased activity of the defense related enzymes, that is, PPO, POD and PAL as well as the total phenols where SA and calcium chloride were the most effective. This could explain potentiality of such compounds to enhance pepper resistance to control *B. cinerea* of the gray mould of pepper (Chen, 2010; Lijuan and Yuxing, 2013). Qin et al. (2003) and Yao and Tian (2005) found that exogenous SA could significantly induce -1, 3-glucanase, phenylalanine ammonia-lyase (PAL) and peroxidase (POD) and effectively restrain pathogen expansion of *Monilinia fructicola* in harvested sweet cherry fruit. These studies proved the important role of exogenous SA in activating fruit defense responses against fungal pathogen infection. However, up to date, most experiments of SA-mediated defense response of fruit are more concerned in hydrolytic enzymes and PR-proteins (Ding et al., 2001, 2002; Qin et al., 2003; Yao and Tian, 2005). There are some different methods used to reduce discoloration of fruits and inhibition microbial growth by reducing moisture losses, limitation of oxygen uptake, inhibition of respiration, retardant ethylene production, these methods are SA

application, fruit dipping in calcium treatments, safe edible coating and also, modified atmosphere (Fisk et al., 2008; Yao and Tian, 2005; Montanaro et al., 2006). Meantime, diameter of the rotting area decreased with decreasing storage temperature and vice versa where 8°C exhibited the least diameter of gray mould between 8 and 25°C. Meanwhile, calcium chloride (1.5 g/L) and SA (1 mM) exhibited a further effect to decrease the developed gray mould under the different degrees of storage temperature. Moreover, calcium chloride effectively delayed time of appearance of the first signs of gray mould (that is, increased shelf life) of the inoculated pepper fruits of both tested cultivars as there were no gray mould symptoms up to seven days after inoculation with *B. cinerea* (*BC-3* isolate).

The obtained results were in harmony with Sakaldaş and Kaynaş (2010) and Gross et al. (2004). Calcium was employed for harvested fruits in preserving qualities, controlling softening, inhibiting the ratio of rottenness and extending shelf life (Chen et al., 2011). Calcium dips have been employed to improve firmness and extend the postharvest shelf life of a wide range of fruits and vegetables (Hernandez-Munoz et al., 2006). Pre- and postharvest application of calcium may help to reduce senescence during commercial and retail storage of fruit, with no detrimental effect on consumer acceptance (Lester and Grusak, 2001). Pila et al. (2010) reported that the application of calcium prolonged the storage life of strawberries and tomato fruits, respectively, as measured by a delay in accumulation of sugars, decrease in organic acids, increase of colour saturation index and mold development. Further, Lam et al. (1987) stated that SA as an antitranspirant chemical can retard moisture loss associated pericarp browning of fruits. Senescent changes resulting to losses in physicochemical changes and nutritional qualities can also be inhibited. Consequently, fruit storage life could be prolonged. The post-harvest application of $CaCl_2$ was reported to increase the storage shelf life of apricots (Antunes et al., 2003), Kiwi (Dimitrios and Pavlina, 2005; Arhtar et al., 2010), peaches (Manganaris et al., 2007), tomato (Pila et al., 2010) and strawberries (Verdini et al., 2008). This effect was mainly by reducing pectin solubility (Lara et al., 2004), strengthening cell wall (Vicente et al., 2007), maintaining firmness (Manganaris et al., 2007), and delaying fruit ripening and decreasing decay rate (Lara et al., 2004). In the light of these results, we recommend the use of SA (1 mM) and $CaCl_2$ (1.5 g/L) treatments to maintain the quality of peppers during postharvest storage.

Therefore, commercial application of SA (1 mM) and $CaCl_2$ (1.5 g/L) can be considered for the maintenance of quality and the extension of shelf life of sweet peppers during storage and marketing. Also, SA and $CaCl_2$ are environmental-friendly method to control the gray mould disease.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Achuo EA, Prinsen E, Hofte M (2006). Influence of drought, salt stress and abscisic acid on the resistance of tomato to *Botrytis cinerea* and *Oidium neolycopersici*. Plant Pathol. 55:178-186.

Alazem M, Lin KY, Lin NS (2014). The abscisic acid pathway has multifaceted effects on the accumulation of Bamboo mosaic virus. Mol. Plant Microbe. Interact 27:177-189.

Anesini C, Ferraro GE, Filip R (2008). Total polyphenol content and antioxidantcapacity of commercially available tea (*Camellia sinensis*) in Argentina. J. Agric.Food Chem. 56:9225-9229.

Antunes MD, Correia C, Miguel MP, Martins MG, Neves MA (2003).The effect of calcium chloride postharvest application on fruit storage ability and quality of 'Beliana' and 'Lindo' apricot (*Prunus armeniaca* L.) cultivars. Acta Horticulturae. (604):721-726.

Arhtar A, Abbasi NA, Hussain A (2010). Effect of calcium chloride treatments on quality characteristics of loquat fruit during storage. Pak. J. Bot., 42:181-188.

Asselbergh B, Curvers K, Franca SC, Audenaert K, Vuylsteke M, Van Breusegem F, Hofte M (2007). Resistance to *Botrytis cinerea* in sitiens, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. Plant Physiol. 144:1863-1877.

Audenaert K, De Meyer GB, Hofte M (2002). Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. Plant Physiol. 128: 491-501.

Babalare M, Asghari M, Talaei A, Khosroshahi A (2007). Effect of pre- and postharvest salicylic acid treatment on ethylene production, fungal decay and overall quality of Selva strawberry fruit, Food Chem. 105:449-453.

Balogun OS, Odeyemi GA, Fawole OB (2005). Evaluation of the pathogenic effect of some isolates on fruits and seedlings of pepper (*Capsicum spp*). Agric. Res & Dev. 2:159-169.

Cao SF, Hu ZC, Zheng YH, Lu BH (2010. Synergistic effect of heat treatment and salicylic acid on alleviating internal browning in cold-stored peach fruit, Postharvest Biol. Technol. 58:93-97.

Chen C, Belanger RR, Benhamou N, Paulitz TC (2000). Defense enzymes induced in cucumber roots by treatments with plant growth promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. Physiol Mol Plant Pathol. 56: 13-23.

Chen NL, Hu M, Dai CY, Yang SM (2010). The effects of inducing treatments on phenolic metabolism of melon leaves. Acta Hort. Sinica, 37:1759-1766.

Chen F, Liu H, Yang H, Lai S, Cheng X, Xin Y, Yang B, Hou H, Yao Y, Zhang S, Bu G, Deng Y (2011). Quality attributes and cell wall properties of strawberries (*Fragaria annanassa* Duch.) under calcium chloride treatment. Food Chem. 126:450-459.

Chittoor JM, Leach HE, White FF (1997). Differential induction of peroxidase gene family during infection of rice by *Xanthomonas oryzae pv. oryzae*. Mol. Plant-Microbe. Interact 10:861-871.

Chittoor JM, Leach JE, White FF (1999). Induction of peroxidase during defense against pathogens. In: Datta, S.K., Muthukrishnan, S. (Eds.), Pathogenesis: Related Proteins in Plants. CRC Press, Boca Raton, FL, P 291.

Damalas CA, Eleftherohorinos IG (2011). Pesticide Exposure, Safety Issues, and Risk Assessment Indicators. Int. J. Environ. Res. Public Health. 5:1402-1419.

Darras AI, Terry LA, Joyce DC (2005). Methyl jasmonate vapour treatment suppresses specking caused by Botrytis cinerea on cut *Freesia hybrida L.* flowers. Postharvest Biol. Technol. 38:175-182.

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J (1994). A central role of salicylic acid in plant disease resistance. Science 266: 1247-1250.

Denancé N, Sánchez-Vallet A, Goffner D, Molina A (2013). Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. Front Plant Sci. 4:1-12.
Dickerson DP, Pascholati SF, Hagerman AE, Butler LG, Nicholson RL (1984). Phenylalanine ammonia-lyase and hydroxyl cinnamate: CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. Physiol. Plant Pathol. 25:111-123.
Dimitrios G, Pavlina DD (2005). Summer-pruning and preharvest calcium chloride sprays affect storability and low temperature breakdown incidence in kiwifruit. Postharvest Biol. Technol. 36:303-308.
Ding CK, Wang CY, Gross KC (2002). Jasmonate and salicylate induce the expression of pathogenesis-related-protein genes and increase resistance to chilling injury in tomato fruit. Planta 214:895-901.
Ding CK, Wang CY, Gross KC, Smith DL (2001). Reduction of chilling injury and transcript accumulation of heat shock proteins in tomato fruit by methyl jasmonate and methyl salicylate. Plant Sci. 161:1153-1159.
Elad Y, Shtienherg D (1995). *Botrytis cinerea* in greenhouse vegetables; chemical, cultural, physiological and biological controis and their integration. Integ Pest Manag. Rev 1:15-29.
Elad Y, Williamson B, Tudzynski P, Delen N (2004). *Botrytis spp*. and diseases they cause in agricultural systems – an introduction. *Botrytis*: Biology, Pathology and Control (Elad Y, Williamson B, Tudzynski P & Delen N, eds), pp. 1–6. Kluwer Academic Publishers, Dordrecht, the Netherlands.
El-Gali ZI (2008). Control of decay of apple fruits used by Calcium and Sodium chloride salts. Al-Mukhtar J. Sci. 20 97-111.
El-Hifny MM, Islah MA, El-Sayed M (2011). Response of Sweet Pepper Plants Growth and productivity by application of ascorbic acid and biofertilizers under saline condition. Austr. J. Basic Appl. Sci. 5-6:1273.
Fisk CL, Silver AM, Strik BC, Zhao Y (2008): Postharvest quality of hardy Kiwifruit (*Actinidia arguta* Ananasnaya) associated with packaging and storage conditions. Postharvest Biol. Technol. 47:338-345.
Godoy G, Steadman JR, Dickman MB Dam R (1990). Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia* sclerotiorum on *Phaseolus vulgaris*. Physiol. Mol. Plant Pathol. 37:179-181.
Gomez KA, Gomez AA (1984). Statistical procedures for agricultural research. John Wiley Sons, Inc., New York.
Gross KC, Wang CY, Saltveit M (2004). The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks. Agriculture Handbook Number 66. USDA, ARS.
Hajhamed AA, Abd El-Sayed WM, Abou El-Yazied A, Abd-El-Ghaffar NY (2007). Suppression of bacterial soft rot disease of potato. Egyptian J. Phytopath. 35-2:69-80.
Hernandez-Munoz P, Almenar E, Ocio MJ, Gavara, R (2006). Effect of calcium dips and chitosan coatings on postharvest life of strawberries (*Fragaria ananassa*). Postharvest Biol. Technol. 39:247-253.
Houeto PG, Bindoula, Hoffman JR (1995). Ethylene bisdithio carbamates and Ethylene ethiourea: possible human health hazards. Environ. Health Perspect. 103:568-573.
Ijato JY, Otoide JE, Ijadunola JA, Aladejimokun AO (2011). Efficacy of antimicrobial effect of *Vernonia amygdalina* and *Tridax procumbens* in *in vitro* control of tomato (*Lycopersicum esculentum*) post harvest fruit rot. Rep. Opin. 3-1:120-123.
Huang RH, Liu JH, Lu YM, Xia RX (2008). Effect of salicylic acid on the antioxidant system in the pulp of 'Cara cara' navel orange (*Citrus sinensis* L Osbeck) at different storage temperatures, Postharvest Biol. Technol. 47:168-175.
Jiankang C, Kaifang Z, Weibo J (2006). Enhancement of postharvest disease resistance in Ya Li pear (*Pyrus bretschneideri*) fruit by salicylic acid sprays on the trees during fruit growth, Europ. J. Plant Pathol. 114:363-378.
Jin P, Zheng YH, Tang SS, Rui HJ, Wang CY (2009). Enhancing disease resistance in peach fruit with methyl jasmonate. J. Sci. Food Agric. 89:802-808.
Karabulut OA, Gabler FM, Mansour M, Smilanick JL (2004). Postharvest ethanol and hot water treatments of table grapes to control gray mould. Postharvest Biol. Technol. 34:169-177.
Kiran KS, Linguratu, Adiver S (2006). Effect of plant extract on *Sclerotium rolfsii*, the incitant of stem rot of groundnut .J. Mycol. Plant Pathol. 36:77-79.
Kim DS, Hwang BK (2014). An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signaling of the defence response to microbial pathogens. J Exp Bot. 65:2295-2306.
Kolattukudy PE, Mohan R, Bajar MA, Sherf BA (1992). Plant peroxidase gene expression and function. Biochem. Soc. Trans. UK 20: 333-337.
Lara I, García P, Vendrell M (2004). Modifications in cell wall composition after cold storage of calcium-treated strawberry (Fragaria × ananassa Duch.) fruit. Postharvest Biol. Technol. 34:331-339.
Lattanzio V, Lattanzio VMT, Cardinali A (2006). Role of phenolics in the resistance mechanism of plants against fungal pathogens and insects. In: Imperato F, ed. Phytochemistry: advances in research. Trivandrum, Kerala, India: Research Signpost, pp. 23-67.
Lebda A, Luhová L, Sedlářová M, Jančová D (2001). The role of enzymes in plant – fungal pathogens interactions. J. Plant Dis. Port. 108:89-111.
Leon AT, Daryl JC (2004). Elicitors of induced disease resistance in postharvest horticultural crops: a brief review. Postharvest Biol. Technol. 32:1-13.
Lester GE, GrusakMA (2001). Postharvest application of chelated and nonchelated calcium dip treatments to commercially grown honey dew melons: effects on peel attributes, tissue calcium concentration, quality, and consumer preference following storage. HortTechnology 11:561-566.
Li L, Steffens JC (2002). Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. Planta, 215:239-247.
Lijuan G, Yuxing Z (2013). Effect of Salicylic Acid on Pear Leaf Induced Resistance to Pear Ring Rot. World Appl. Sci. J. 22(11):1534-1539.
Manganaris GA, Vasilakakis M, Diamantidis G, Mignani (2007). The effect of postharvest calcium application on tissue calcium concentration, quality attributes, incidence of flesh browning and cell wall physicochemical aspects of peach fruits. Food Chem. 100:1385-1392.
Maxwell DP, Lumsden RD (1970). Oxalic acid production by *Sclerotinia sclerotiorum* in infected bean and in culture. Phytopathology 60:1395-1398.
Mayer AM, Harel E, Shaul RB (1965). Assay of catechol oxidase: a critical comparison of methods. Phytochemistry 5:783-789.
Meng XH, Li BQ, Liu J, Tian SP(2008). Physiological responses and quality attributes of table grape fruit to chitosan preharvest spray and postharvest coating during storage. Food Chem. 106:501-508.
Meng XH, Han J, Wang Q, Tian SP(2009). Changes in physiology and quality of peach fruits treated by methyl jasmonate under low temperature stress. Food Chem. 114:1028-1035.
Mengiste T, Laluk K, Abu Qamar S (2010). Mechanisms of induced resistance against *B. cinerea*. In Postharvest Pathology (Prusky, D. and Lodovica-Gullino, M., eds). London: Springer. pp. 13-30.
Mohr PG, Cahill DM(2003). Abscisic acid influences the susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae pv tomato* and *Peronospora parasitica*. Funct. Plant Biol. 30 461-469.
Mohr PG, Cahill DM (2007). Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes in Arabidopsis infected with *Pseudomonas syringae pv. tomato*. Funct. Integr. Genom. 7:181-191.
Montanaro G, Dichio B, Xiloyannis C, Celano G (2006): Light influences transpiration and calcium accumulation in fruit of Kiwifruit plants (*Actinidia deliciosa* var. deliciosa). Plant Science 170:520-527.
Murphy AM, Carr JP (2002). SA has cell-specific effects on Tobacco mosaic virus replication and cell-to-cell movement. Plant Physiol. 128:552-563.
Naradisorn M (2013). Effect of calcium nutrition on fruit quality and postharvest diseases. Intern. J. Sci. Innov. Discov. 3-1:8-13.
Nsabiyera V, Ochwo-ssemakula M, Sseruwagi P (2012). Hot pepper

reaction to field diseases. Afr. Crop Sci. J. 20 (1):77-97.
Nie X (2006). Salicylic acid suppresses Potato virus Y isolate N: Oinduced symptoms in tobacco plants. Phytopathol., 96: 255-263.
Pernezny K, Roberts PD, Murphy JF, Goldberg NP (2003). Compendium of pepper diseases. APS Press, P 63.
Pieterse CM, Leon-Reyes A, Van der Ent S and Van Wees SC (2009). Networking by small-molecule hormones in plant immunity. Nature Chem Biol. 5:308-316.
Pila N, Gol NB, Rao TVR (2010). Effect of postharvest treatments on physicochemical characteristics and shelf life of tomato (*Lycopersicon esculentum* mill.) fruits during storage. Am. Eura. J. Agric. Environ. Sci. 9:470-479.
Zheng PYH, Tang SS, Rui HJ, Wang CY (2009). Enhancing disease resistance in peach fruit with methyl jasmonate. J. Sci. Food Agric. 89:802-808.
Qin GZ, Tian SP, Xu Y, Wan YK (2003). Enhancement of biocontrol efficacy of antagonistic yeasts by salicylic acid in sweet cherry fruit. Physiol. Mol. Plant Pathol. 62:147-154.
Ozdemir M, Floros JD (2004). Active Food Packaging Technologies. CRC Crit. Rev. Food Sci. Nutrit. 44-3:185-193.
Okigbo RN, (2009). Variation in phytochemical properties of selected fungicidal aqueous extract of some plant leaves in Kogi State, Nigeria. American-Eurasian J. Sust. Agric. 3(3):407-409.
Ramezanian A, Rahemi M (2007. Effect of Pre-storage application of spermidine, calcium chloride and hot water on chilling injury of cold storage pomegranate Acta Horticulturae, 877:4-50.
Rao TV, Gol RNB, Shah KK (2011). Effect of postharvest treatments and storage temperatures on the quality and shelf life of sweet pepper (*Capsicum annum* L.) Scientia Horticulturae .132:18-26.
Raposo R, Colgan R, Delcan J, Melgarejo P (1995). Application of an automated quantitative method to determine fungicide resistance in *Botrytis cinerea*. Plant Dis. 79-3:294-296.
Raskin I (1992). Role of salicylic acid in plants. Annu Rev Plant Physiol? resistance in peach fruit with methyl jasmonate. J. Sci. Food Agric. 89:802-808.
Rosslenbroich HJ, Stuebler D (2000). *Botrytis cinerea* history of chemical control and novel fungicides for its management. Crop Protect. 19:557-561.
SAS Institute. (2009). SAS/STAT 9.2 Users Guide. SAS Institute, Cary.
Sakaldaş M, Kaynaş K (2010). Biochemical and quality parameters changes of green sweet bell peppers as affected by different postharvest treatments. Afr. J. Biotech., 9(48):8174-8181.
Sayyari M, Babalar M, Kalantari S, Serrano M, Valero D (2009). Effect of salicylic acid treatment on reducing chilling injury in stored pomegranates, Postharvest Biol. Technol., 53: 152-154
Srivastava MK, Dwivedi UN (2000). Ripening of banana fruit by salicylic acid, Plant Sci. 158:87-96.
Thaler SJ, Bostock RM (2004). Interactions between abscisic-acid mediated responses and plant resistance to pathogens and insects. Ecology 85:48-58.
Thilagavathi R, Saravanakumar D, Ragupathi N, Samiyappan R (2007). A combination of biocontrol agents improves the management of dry root rot (*Macrophomina phaseolina*) in greengram. Phytopathol. Mediterr. 46:157-167.
Vagelas AP, Kalorizou H, Wogiatzi E (2009). Biological control of *Botrytis* fruit rot (gray mould) on strawberry and red pepper fruits by olive oil mill wastewater. Biotechnol. & Biotechnol. pp. 1489-1491.
Vale FXR, Zambolim L, Paul PA, Costa H (2000). Doenças causadas por fungos em tomate. In: Zambolim L, Vale FXR, Costa H (Eds.) Controle de doenças de plantas: hortaliças. Viçosa MG. Suprema Gráfica e Editora. pp. 699-756.
Verdini RA, Zoggilla SE, Rubiolo A (2008). C. Calcium uptake during immersion of strawberries in $CaCl_2$ solutions. J. Food Sci. 73:533-539.
Vicente AR, Saladié M, Rose JK, Labavitch CJM (2007). The linkage between cell metabolism and fruit softening: Looking to the future. J. Sci. Food Agric. 87:1435-1448.
Xu X, Tian S (2008). Salicylic acid alleviated pathogen-induced oxidative stress in harvested sweet cherry fruit. Postharvest Biol. Technol. 49:379-385.
Yao HJ, Tian SP, Qin G, Liu Z (2005). Effects of pre-and post-harvest application of salicylic acid or methyl jasmonate on inducing disease resistance of cherry fruit in storage. Postharvest Biol. Technol. 35:253-262.
Yaqub F, Shahzad S (2006). Effect of fungicides on *in vitro* growth of *Sclerotium rolfsii*. Pak. J. Bot. 38(3):881-883.
Yang DL, Yang Y, He Z (2013). Roles of plant hormones and their interplay in rice immunity. Mol. Plant 6: 675-685.
Yedidia I, Benhamou N, Kapulnink Y, Chet I (2000). Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *Trichoderma harzianum* strain T-203. Plant Physiol. Biochem. 38: 863-873.
Wang LJ, Chen SJ, Kong WF, Li SH, Archbold DD (2006). Salicylic acid pretreatment alleviates chilling injury and effects the anti-oxidant system and heat shock proteins of peaches during cold storage, Postharvest Biol. Technol. 41:244-251.
Wasternack C, Hanse B (2013). Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. Ann. Bot. 111: 1021-1058.
Zhang Z, Liu X, Hong X (2007). Effects of home preparation on pesticide residues in cabbage. Food Control 18 (12), 1484–1487.
Zhou ML, Yang XB, Zhang Q, Zhou M, Zhao EZ, Tang YX, Zhu XM, Shao JR, Wu YM(2013). Induction of annexin by heavy metals and jasmonic acid in *Zea mays*. Funct. Integr. Genomics 13: 241-251.
Zhou T, Boland GJ (1999). Mycelial growth and production of oxalic acid by virulent and hypovirulent isolates of *Sclerotinia sclerotiorum*. Canadian J. Plant Path. 21:93-99.
Zieslin N, Ben–Zaken R (1993). Peroxidase activity and presence of phenolic substances in peduncles of rose flowers. Plant Physiol. Biochem. 31: 333-339.
Zitter TA (2011). Pepper disease control. Available at: http:// vegetablemdonline.ppath.cornell.edu.

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