Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9283
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v35i1.8612

Comparative electrophoretic profile of proteins and esterases in healthy silkworm larvae (*Bombyx mori* Lineu, 1758) and infected with nucleopolyhedrovirus

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ABSTRACT. Total proteins and esterases from silk gland extracts of *Bombyx mori* silkworm were characterized and compared with electrophoretic profiles of prepared extracts with silkworm glands infected with nucleopolyhedrovirus (BmNPV). SDS-PAGE (7%) gels was used for the total proteins, and 10% native PAGE for esterases. In the silk glands extracts of healthy silkworms, it was observed seven protein zones with molecular weight varying between 10 kDa (P1) and 60 kDa (P7). In the infected silkworms, a new zone named P8 (90 kDa) was also detected. Esterases activity at 5th instar larvae underwent changes after the infection with BmNPV, since there was a reduction (EST-6 and EST-7) and an increase (EST-8) in the intensity of the regions of esterases activity, and specificity of EST-9 to β-naphthyl acetate. Those alterations observed in the expression of genes after the infection with the nucleoplyhedrovirus can be used as markers to detect infections in *B. mori*.

Keywords: viral infection, silk gland, isoenzymes, SDS-PAGE, molecular markers.

Perfil eletroforético comparativo de proteínas e esterases em lagartas de bicho da seda (*Bombyx mori* Lineu, 1758) saudáveis e infectadas por nucleopoliedrovírus

RESUMO. Nesse estudo foram identificadas e comparadas as alterações das proteínas totais e esterases em extratos de glândula sericígena de lagartas de *Bombyx mori* sadias e infectadas por nucleopoliedrovírus (BmNPV), empregando eletroforese SDS-PAGE com géis a 7% para proteínas totais, e PAGE a 10% para esterases. Nos extratos de glândulas sericígenas de lagartas saudáveis, foram observadas sete regiões proteicas com peso molecular que variam entre 10 kDa (P1) a 60 kDa (P7). Naquelas infectadas pelo BmNPV, uma nova região denominada de P8 (90 kDa) foi detectada. A atividade das esterases no 5º instar larval sofreu alterações após a infecção pelo BmNPV porque houve redução (EST-6 e EST-7) e aumento (EST-8) na intensidade das regiões de atividade de esterases; e especificidade da EST-9 com o substrato β-naftil acetato. Essas alterações na expressão gênica, após a infecção pelo nucleopoliedrovírus, poderão ser utilizadas como marcadores para detecção da infecção em *B. mori*.

Palavras-chave: infecção viral, glândula de seda, isoenzimas, SDS-PAGE, marcadores moleculares.

Introduction

Some insects produce a set of proteins when stimulated by microbial infection (BOMAN, 1991). According to Shiotsuki and Kato (1999) some proteins involved in the detoxification can be induced by microbial infection, such as esterase, and may also help eliminate toxic molecules generated during microbial infection. These authors found two bacterially inducible carboxylesterases (CEs) in the silkworm's hemolymph, *Bombyx mori*. EST-1 and EST-2 were induced by lipopolysaccharide injection after 6 hours, as well as *E. coli* infection. Those bacterially inducible CEs clearly differed from the noninducible CEs, including juvenile hormone esterases.

Bombyx mori nucleopolyhedrovirus (BmNPV) is the most significant virus in the sericultural industry, often causing severe economic damages (PONNUVEL et al., 2003). The baculovirus life cycle is peculiar because it produces two types of infectious progenies with different functions, but essential to natural propagation. The occluded form of the virus (PDV) is responsible for the transmission from insect to insect, while the non-occluded form (BV) is responsible for transmission from cell to cell, in a same individual (systemic infection) (GRANADOS; FEDERICI, 1986).

According to Castro et al. (1999) after 80 to 100 hours of infection, there is the discoloration of insect's tegument in several hosts. After death, the insect usually breaks up easily, releasing great amount of polyhedrons into the atmosphere, which in turn will infect new larval populations of the host insect. The baculovirus produces some proteins that aid in the infectious process. The chitinase (HAWTIN et al., 1995) and cysteine-protease (OHKAWA et al., 1994) are proteins secreted in the late phase of the cellular infection, getting accumulated in the host larvae as the infection progresses. Probably they act in the dissolution of the insect tissues, more specifically in the larval cuticle that breaks up after the death of the host, releasing the polyhedrons (HAWTIN et al., 1997).

Ponnuvel et al. (2003) found a digestive lipase (Bmlipase-1) enzyme with potential to operate as a physiological barrier against BmNPV at the initial site of viral infection.

Enhanced locomotory activity (ELA) is a normal behavior that occurs at the end of the larval stage in Lepidoptera. Baculovirus infection can also induce ELA in lepidopteran larvae. The molecular mechanisms of this behavior are not fully understood. Kamita et al. (2005) showed that a baculovirus encoded protein tyrosine phosphatase (PTP) gene (*ptp*) induces ELA. These authors proposed that the modern baculovirus may have acquired *ptp* gene from an ancestral host and that this gene was selectively maintained because it increases virus transmission. These results support the Goulson (1997) hypothesis.

A comparison between the proteomes of these three silkworm strains (highly resistant, susceptible and isogenic hybrid) led to the identification of two differentially expressed proteins, beta-N-acetylglucosaminidase 2 and aminoacylase. The expression levels of these proteins were higher in the BmNPV resistant strains (LIU et al., 2010).

Shirata et al. (2010) carried out the identification and characterization of cellular and/or viral factors that are responsible for the host specificity determination of baculoviruses. The analyses also provide insights into baculovirus genomic alterations due to gene acquisitions and losses that are involved in the coevolution of baculoviruses and insect cells (SHIRATA et al., 2010). The authors demonstrated that *hycu-ep32* gene encoded by the HycuNPV genome is involved in restricted BmNPV multiplication in BmN-4 cells.

The identification and characterization of behavioral changes and gene expression in larvae of *B. mori* infected with nucleopoliedovirus have been made, but there is not a fast and safe way to diagnose the infection early.

This study was carried out to identify and compare the alterations of the total proteins and esterases in larvae silk gland of healthy and infected with nucleopolyhedrovirus *B. mori*. Gene expression changes after the infection may be used as markers to detect the nucleopolyhedrovirus presence in silkworm.

Material and methods

Insect treatment

Healthy and infected with nucleopolyhedrovirus (BmNPV) *B. mori* larvae at 5th instar (with more developed silk glands) were used. Silkworm breeders from northwestern Paraná State have provided 300 healthy silkworms (hybrids of the Japanese and Chinese lines). One hundred and fifty (150) larvae were sacrificed and stored at -20^{o}C. The other 150 larvae were used for the production of infected larvae. The silk glands of 300 larvae (150 healthy and 150 infected) were submitted to PAGE and SDS-PAGE electrophoresis.

Ten silkworms (5th instar) infected with BmNPV (collected from Alto Piquiri, Paraná State) were sacrificed, homogenized and spread on the mulberry leaves that fed the 150 healthy larvae in a period of 3 to 4 days. The infected larvae were sacrificed and stored at -20^{o}C.

The larvae silk glands were removed and homogenized in 150 μL of the extraction solution (2-mercaptoethanol 0.1% and 50 μL of carbon tetrachloride). The samples were centrifuged for 30 minutes at 25,000 rpm at 2^{o}C.

Polyacrylamide electrophoresis

Native vertical polyacrylamide gel electrophoresis (PAGE) was undertaken with 1.0 mm polyacrylamide gels (10%) for esterases analysis. Staining for esterase activity was performed using α and β-naphthyl acetate as substrate in the presence of Fast Blue RR Salt, as a color-developing reagent according to Lapenta et al. (1995).

SDS-PAGE vertical polyacrylamide gel electrophoresis was carried out with 1.0 mm polyacrylamide gels (7%) for soluble proteins analysis. The peptides visualization was accomplished by staining in Coomassie Blue solution (10%) in fixative PAGE for 12 hours and in bleaching solution (5 water: 1 ethanol: 1 acetic acid) until removing the excessive dye. Marker Benchmark (Invitrogen molecular weight of 10-220 kDa) was used.

The esterases and peptides were named according to their electrophoretic mobility; the more cathodic was named EST-1 and P1 respectively.

Results and discussion

Total protein characterization

Seven protein zones were observed in the silk glands of healthy larvae analyzed by SDS-PAGE, with molecular weight varying between 10 kDa (P1) and 60 kDa (P7). In silk glands of larvae infected with nucleopolyhedrovirus, it was observed an additional zone, P8, with 90 kDa of molecular weight and remarkable reduction in the P7 intensity (Figure 1).

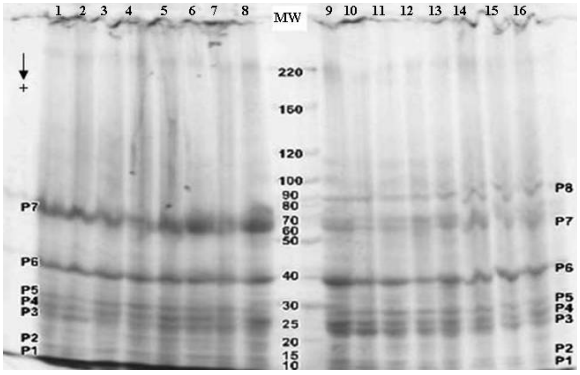

Figure 1. Total proteins profile of the 5th instar silkworm larvae by SDS-PAGE (7%) - Coomassie Blue staining. Numbers 1-8 = silk gland of healthy larvae; 9-16 = silk gland of larvae infected with nucleopolyhedrovirus. MW = Molecular weight (KDa).

Ohkawa et al. (1994) sequenced and analyzed a functionally active BmNPV cysteine protease gene whose deduced sequence of amino acids has significant homology to those of the papain family of cysteine proteases. This was the first report of a virus-encoded protease with activity on general substrates and evidences that a virus-encoded protease may play a role in degradation of infected larvae to facilitate horizontal transmission of the virus.

Morris and Miller (1992) found that the different virus promoters have influence on the expression of heterologous gene in several cellular lineages of numerous insects (*Spodoptera frugiperda* IPLB-SF21, *Choristoneura fumiferana* Clemens IPL-CF-1, *Mamestra brassicae* L. Cathedral-MaBr-3, *B. mori* L. BmN-4, disparate L. IPLB-LD652Y, *Helicoverpa zea* Bopddie Hz1b3 and *Drosophila melanogaster* Schneider), for replication of AcNPV. These authors suggested that the specific differences that prevent permissive infections are peculiar for each cellular lineage. The infection analyses in cells of *B. mori* were non permissive, revealing differences in viral DNA replication and in the use pattern of the promoter in each cellular lineage, suggesting a variety of obstacles that prevent the infection.

In all samples from infected larvae it was observed the protein P8. This result suggests that P8 can become a good biochemical marker for detecting the presence of the virus in silkworm breeding. However, qualitative and quantitative studies on that peptide zone will be necessary for a better understanding of its expression and function in the silkworm.

Esterases characterization

Eight zones of esterase activity were observed in the silk glands of both healthy larvae and infected with BmNPV (Figure 2). All of the eight esterases are αß-esterases, because they were stained by both substrates applied.

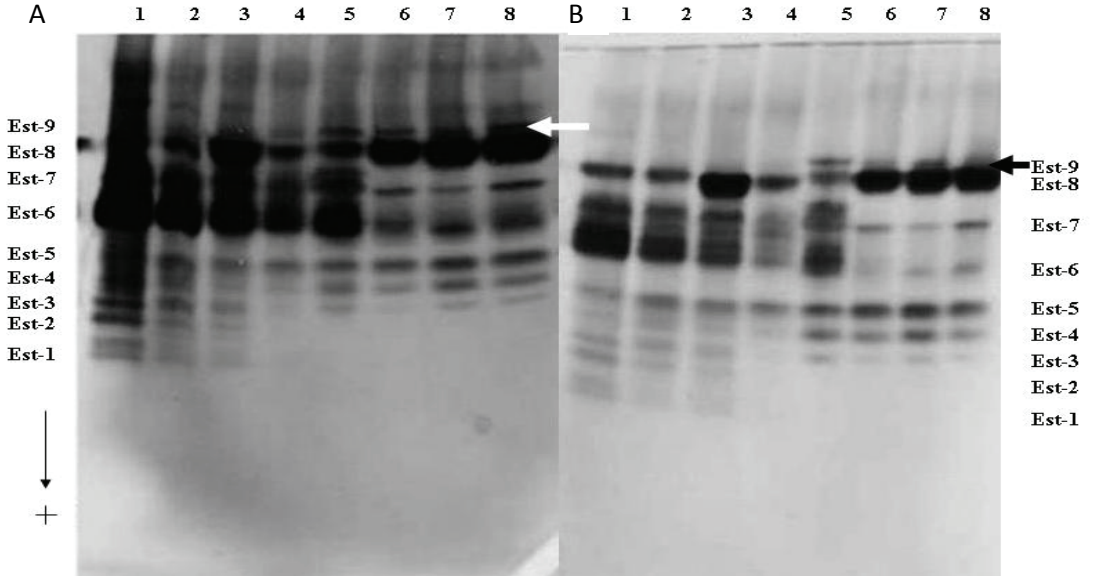

Figure 2. Esterases patterns in extracts of silk glands larvae of 5th instar of healthy *B. mori* (numbers 1 to 4) and infected with BmNPV (numbers 5 to 8). A = α-naphthyl-acetate and B = β-naphthyl-acetate. ⋆The arrow indicates the new esterase after infection with BmNPV.

EST-1 and EST-2 were not detected in 5th instar larvae after infection with BmNPV (Figure 2).

In silk glands extracts of healthy and infected larvae no differences were observed in EST-3, EST-4 and EST-5, but a decrease was detected in the intensity of EST-6 and EST-7 isoenzymes in all analyzed larvae. Although being just qualitative data, those results showed that may have occurred a decrease in the synthesis of these proteins, leading to a reduction in the staining intensity of esterases.

In contrast, an intensity increase in EST-8 band was observed in infected larvae, leading to an increase in the synthesis of this enzyme.

In Figure 2 it can be observed that EST-9 showed differences in the substrate affinity for α and β naphthyl acetate. When stained with α-naphthyl-acetate, both infected and uninfected samples have showed the EST-9, whereas when stained with β-naphthyl-acetate, the EST-9 was observed only in infected samples. This result was observed in silk glands extracts of all infected larvae (150) analyzed. We suggest that the nucleopolyedrovirus infection probably changes the molecular structure of EST-9 by changing its catalytic site and affinity for substrate.

After the BmNPV infection, the cellular metabolism of *B. mori* becomes totally altered and mobilized for the production of viral particles. As a consequence, there is a modification in the synthesis and chemical components of the procuticle secretion that loses its structural integrity (BRANCALHÃO; RIBEIRO, 2003).

The viral infection can completely change the host protein synthesis, preventing and/or starting the protein synthesis depending on the viral infection cycle to be synthesized. The synthesis and concentration of intracellular enzymes are under nuclear genes control. The molecules inside the cell can stimulate or inhibit the specific enzyme activity.

The infection with BmNPV in *B. mori* larvae promoted an alteration in the synthesis of some esterases in those insects. That fact can be observed by the absence of EST-1 and EST-2, probably, due to inhibition of the expression of the genes of those esterases.

The viral infection can modify the synthesis of host proteins, because the expression of viral genes can change the genes expression of the host. Bose et al. (1997) studied the regulation of *vaccinia virus* infection using three animal cell lines. They verified that the rate of synthesis of a major host protein in infected cells measured after two hours of viral infection declined more than 50%. The rate of synthesis of host proteins in viral-resistant cells has remained unchanged.

Isoenzyme mobility differences seem to be restricted to specific tissue (MURPHY; CRABTREE, 1985), or are function of environmental conditions and/or physiological state of the organism (MCGOVERN; TRACY, 1981). Isoenzyme complex patterns observed can be explained by the action of different genetic and epigenetic mechanisms (HARRIS, 1966).

Liu and Bilimoria (1997) have studied the infection with BmNPV in *Spodoptera frugiperda*. They observed that the inhibition of host protein synthesis only occurred in productive infections, beginning at 10h post infection (p.i.) and reaching maximal levels around 20h p.i.

The effect of BmNPV infection on the protein metabolism of the silkworm (*B. mori*) larvae was studied by Etebari et al. (2007). Their results have demonstrated that the total protein decreased considerably in infected larvae compared to control. This reduction was observed in both sampling times, so that the total protein in the third day of 5th instar was 36.5 mg mL^{-1} in healthy larvae, while in the same day; the amount of protein in infected larvae was 7.5 mg mL^{-1}. This trend continued through the fifth day of 5th instar, and the amount of total protein in infected larvae decreased to 50% of the control.

Baculovirus infection starts when an occlusion body is taken in by the sensitive insects. Midgut fluid of lepidopteran larvae is totally alkali and digests the viral occlusion bodies. Consequently, virions are released into the alimentary system and cross the peritrophic membrane. They combine with midgut epithelial cells and enter into the nuclei, starting the first cycle of viral production and replication. These processes cause many biochemical changes in larvae, which respond to these biological phenomena by changing many of its metabolisms to defend themselves against pathogen invasion (ETEBARI et al., 2007). The understanding and identifying these biochemical changes will be very important for discussing many biological stresses. The determination of the biochemical responses in silkworm against BmNPV could facilitate the control of agricultural pests (GAO et al., 2006).

The isoenzymes EST-6, EST-7, EST-8 and EST-9 can become molecular markers for the detection of the infection with BmNPV in *B. mori*. However, subsequent studies are needed to verify at which instar the expression of esterases genes starts to be altered after the infection, and which of these isoesterases would be the best marker for detecting the infection with BmNPV.

Conclusion

The peptide P8 can be used as a biochemical marker to detect the nucleopolyhedrovirus presence (BmNPV) in *B. mori* cultures. This peptide shows that there were alterations in the synthesis of protein after the infection with BmNPV.

The esterases' profile in 5th instar larvae were changed after BmNPV infection. It was observed total inhibition of EST-1 and EST-2 isoenzymes; reduction in the bands intensity of the EST-6 and EST-7 isoenzimes; increase in the bands intensity of EST-8; and detecting EST-9 specificity to β-naphthyl acetate.

Biochemical markers (peptide P8 and EST-6, EST-7, EST-8 and EST-9 isoenzymes) can be used for a fast detection of BmNPV in *B. mori*. Those results can contribute to the knowledge of BmNPV development cycle as compared as silkworm larvae, being useful in the application of prophylactic measures in sericulture. Still, it will be necessary to find out the functional role of both peptide and esterases on the silkworm larvae and on the BmNPV cycle.

References

BOMAN, H. G. Cell-free immunity in *Cecropia*, a model system for antibacterial proteins. **European Journal of Biochemistry**, v. 201, n. 1, p. 23-31, 1991.

BOSE, A.; SAHA, D.; GUPTA, N. K. Viral Infection I. Regulation of protein synthesis during vaccinia viral infection of animal cells. **Archives of Biochemistry and Biophysics**, v. 342, n. 2, p. 362-372, 1997.

BRANCALHÃO, R. M. C.; RIBEIRO, L. F. C. Citopatologia da infecção causada por BmNPV no tegumento de *B. mori* L. 1758 (Lepdoptera: Bombycidae). **Arquivos de Ciências Veterinárias e Zoologia da Unipar**, v. 6, n. 1, p. 15-20, 2003.

CASTRO, M. E. B.; SOUZA, M. L.; SIHLER, W.; RODRIGUES, J. C. M. Biologia Molecular de Baculovírus e seu uso no controle biológico de pragas no Brasil. **Pesquisa Agropecuária Brasileira**, v. 34, n. 10, p. 1733-1761, 1999.

ETEBARI, K.; MATINDOOST, L.; MIRHOSEINI, S. Z.; TURNBULL, M. W. The effect of BmNPV infection on protein metabolism in silkworm (*Bombyx mori*) larva. **Invertebrate Survival Journal**, v. 4, n. 1, p. 13-17, 2007.

GAO, L.; CHEN, K.; YAO, Q.; CHEN, H. BmNPV infection enhances ubiquitin conjugating enzyme E2 expression in the midgut of BmNPV susceptible silkworm strain. **International Journal of Industrial Entomology**, v. 13, n. 1, p. 31-35, 2006.

GOULSON, D. Wipfelkrankheit: modification of host behaviour during baculoviral infection. **Oecologia**, v. 109, n. 2, p. 219-228, 1997.

GRANADOS, R. R.; FEDERICI, B. A. The biology of **baculoviruses**. Florida: CRC Press, 1986. v. 1, p. 89-108.

HARRIS, H. Enzyme polymorphism in man. **Proceedings of the Royal Society of London**, v. 164, n. 995, p. 298-310, 1966.

HAWTIN, R. E.; ARNOLD, K.; AYRES, M. D.; ZANOTTO, P. M. A.; HOWARD, S. C.; GOODAY, G. W.; CHAPPELL, L. H.; KITTS, P. A.; KING, L. A.; POSSEE, R. D. Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. **Virology**, v. 212, n. 2, p. 975-982, 1995.

HAWTIN, R. E.; ZARKOWSKA, T.; ARNOLD, K.; THOMAS, C. J.; GOODAY, G. W.; KING, L. A.; KUZIO, J. A.; POSSEE, R. D. Liquefaction of *Autographa californica* nucleopolyhedrovirus-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. **Virology**, v. 238, n. 2, p. 243-253, 1997.

KAMITA, S. G.; NAGASAKA, K.; CHUA, J. W.; SHIMADA, Y.; MITA, K.; KOBAYASHI, M.; MAEDA, S.; HAMMOCK, B. D. A baculovirus-encoded protein tyrosine phosphatase gene induces enhanced locomotory activity in a lepidopteran host. **Proceedings of the National Academy of Sciences**, v. 102, n. 7, p. 2584-2589, 2005.

LAPENTA, A. S.; BICUDO, H. E. M. C.; CERON, C. R.; CORDEIRO, J. A. Esterase patterns of species in the *Drosophila buzattii* cluster. **Cytobios**, v. 84, n. 336, p. 18-29, 1995.

LIU, H. S.; BILIMORIA, S. L. Regulation of infected-cell-specific protein synthesis in SF-IPLB-21 cells productively infected with *Spodoptera frugiperda* multicapsid nuclear polyhedrosis virus. **Intervirology**, v. 40, n. 1, p. 50-54, 1997.

LIU, X.; YAO, Q.; WANG, Y.; CHEN, K. Proteomic analysis of nucleopolyhedrovirus infection resistance in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). **Journal of Invertebrate Pathology**, v. 105, n. 1, p. 84-90, 2010.

MCGOVERN, M.; TRACY, C. R. Phenotypic variation in electromorphs previously considerad to be genetic markers in *Microtus ochrogaster*. **Oecologia**, v. 51, n. 2, p. 276-280, 1981.

MORRIS, T. D.; MILLER, L. K. Promoter influence on baculovirus-mediated gene expression in permissive and nonpermissive insect cell lines. **The Journal of Virology**, v. 66, n. 12, p. 7397-7405, 1992.

MURPHY, R. W.; CRABTREE, C. B. Evolutionary aspects of isozymes patterns, number of loci, and tissue-specific gene expression in the prairie rattlesnake, *Crotalus viridis viridis*. **Herpetologica**, v. 41, n. 4, p. 451-470, 1985.

OHKAWA, T.; MAJIM, K.; MAEDA, S. A cysteine protease encoded by the baculovirus *Bombyx mori* nuclear polyhedrosis virus. **The Journal of Virology**, v. 68, n. 10, p. 6619-6625, 1994.

PONNUVEL, K. M.; NAKAZAWA, H.; FURUKAWA, S.; ASAOKA, A.; ISHIBASHI, J.; TANAKA, H.; YAMAKAWA, M. A lipase isolated from the silkworm

Bombyx mori shows antiviral activity against nucleopolyhedrovirus. **The Journal of Virology**, v. 77, n. 19, p. 10725-10729, 2003.

SHIOTSUKI, T.; KATO, Y. Induction of carboxylesterase isozymes in *Bombyx mori* by *E. coli* infection. **Insect Biochemistry and Molecular Biology**, v. 29, n. 8, p. 731-736, 1999.

SHIRATA, N.; IKEDA, M.; KOBAYASHI, K. Identification of a *Hyphantria cunea* nucleopolyhedrovirus (NPV) gene that is involved in global protein synthesis shutdown and restricted *Bombyx mori* NPV multiplication in a *B. mori* cell line. **Virology**, v. 398, n. 2, p. 149-157, 2010.

Received on October 30, 2009.
Accepted on August 31, 2011.