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A safe method to control the anthracnose in papaya

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Abstract- This study aimed to propose a safe methodology for the control anthracnose caused by the fungus *Colletotrichum gloeosporioides* in the papaya (*Carica papaya* L.). In addition, yeast present in epiphytic microbiota associated with this fruit were isolated and selected as biocontrol agents, its use in classical and integrated biological control protocols with GRAS substances. For selection as biocontrol agents, the obtained isolates were tested for their ability to: produce antagonistic substances against *C. gloeosporioides*, not grow at 37ºC and subsequently tested for their potential control. Considering the total of 85 isolated yeasts, only UFT 5852 was selected in the above mentioned tests and due to this it was used in biological control tests *in vivo*. It was identified molecularly belonging to species *Anthracocystis grodzinskae* by the sequencing the D1/D2 domain. The results of the biocontrol had showed that the yeast presented a reduction of the disease severity by 93.7%, the sodium bicarbonate 100%, and the biological control integrated with sodium bicarbonate showed a decrease of 84.4%. The treatments did not differ 5% by Tukey test. However, the sodium bicarbonate at 1% showed the best strategy for the control of Anthracnose produced by the phytopathogenic fungus *C. gloeosporioides* in papaya due accessible strategy.
Index terms: epiphytic yeasts, A*nthracocystis grodzinskae, Colletotrichum gloeosporioides*, GRAS substances, biological control.

Método seguro para controle da antracnose em mamão

Resumo - Este estudo teve como objetivo propor uma metodologia segura para o controle da antracnose causada pelo fungo *Colletotrichum gloeosporioides* em mamão *(Carica papaya L.*), além de isolar leveduras presentes, a microbiota epifítica associada a este fruto e selecioná-las como agentes de biocontrole, avaliando seu uso em protocolos de controle biológico clássico e integrado a substâncias GRAS. Para a seleção como agentes biocontroladores, os isolados obtidos foram testados quanto à capacidade de produzirem substâncias antagônicas contra *C. gloeosporioides*, não crescerem à temperatura de 37ºC e, posteriormente, testados quanto a seu potencial de controle. Do total de 85 isolados de leveduras obtidos, apenas o isolado UFT 5852 foi selecionado nos testes acima citados e, com isto, utilizado nos testes de controle biológico *in vivo*. O isolado selecionado foi identificado molecularmente por meio do domínio D1/D2, como pertencente à espécie *Anthracocystis grodzinskae*. Os resultados do controle biológico demonstraram que a levedura apresentou redução de severidade da doença em 93,7%, o bicarbonato de sódio em 100%, e o controle biológico integrado ao bicarbonato de sódio demonstrou redução de 84,4%. Embora a eficiência dos tratamentos com levedura e bicarbonato não tenha diferido entre si ($p<0.05$), bicarbonato de sódio a 1% apresentou ser a melhor estratégia observada para o controle da Antracnose produzida pelo fungo fitopatogênico *C. gloeosporioides* em mamão, devido ser uma estratégia acessível e de baixo custo.
Termos para indexação: leveduras epifíticas, *Anthracocystis grodzinskae*, *Colletotrichum gloeosporioides*, substâncias GRAS, controle biológico.

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Among the diseases that cause significant losses during the post-harvest phase of papaya, Anthracnose stands out, it is caused by *Colletotrichum gloeosporioides*, which is considered one of the most important pathogens related to papaya postharvest and can cause large losses in production (about 90 %) (VALENZUELA et al., 2015).

The disease control is usually performed by the massive use of fungicides, due to its easy applicability, effectiveness and low cost. However, its extensive use can lead to negative consequences such as: the development of resistance to fungicides, toxicity to human health and environmental impacts (LI et al., 2013; VALENZUELA et al., 2015).

For this reason, alternative ways to control Anthracnose have been adopted considering methods that are capable of controlling the disease, that prevent contamination of the fruits by toxic substances and maintain the product quality (GUPTA et al., 2015). In addition, considering the alternative ways that have been used, the biological control agents have been highlighted and several are the studies that indicate that the use of such method has produced positive results and progress over the last two decades (JANISIEWICZ et al., 2010; SPADARO; DROBY, 2016).

Furthermore, among the most commonly used microorganisms for this purpose, yeasts stand out due to the specific characteristics that make them effective as biological control agents, especially in commercial fruits such as papaya (*Carica papaya* L.), because they generally do not produce allergenic or toxic substances, also, they can grow rapidly on inexpensive substrates and are easy to produce in large quantities (SPADARO; DROBY, 2016).

The use of yeasts for fruit postharvest protection has been demonstrated in many studies of biological control. Examples of this satisfactory use of yeasts are: the use of *Hanseniaspora uvarum* in strawberries (CAI et al., 2015), *Saccharomycopsis crataegensis* in oranges (PIMENTA et al., 2010), *Cryptococcus laurentii* and *Candida ciferrii* in apples (VERO et al., 2002). Also, there are several yeast-based products, such as Aspire, which is based on *Candida oleophila*, and Yield Plus, which is based on *Cryptococcus albidus* (JANISIEWICZ et al., 2011), available on the market.

The association of biocontrol yeast to a GRAS substance (Generally Recognized as Safe) allows enhance the control of phytopathogens, thus assisting the biological agents in their antagonistic action in diseases associated with fruits (PIMENTA et al., 2010). These substances are commonly used as food additives, and their uses are sanctioned by the Food Drug Administration (FDA).

Therefore, the combination between classical biological control with GRAS substance enhances the fungistatic or fungicides effects in phytopathogens, with the purpose of increasing the efficiency or the persistence of individual treatments (PALOU et al., 2016).

This study aimed to propose a safe and effective method for the control of anthracnose in papaya fruit, evaluating the potential of isolated yeasts through the epiphytic microbiota of this fruit and GRAS substances against the fungus *C. gloeosporioides*.

Biocontrol agents used in this study were yeast strains isolated from the surface of 20 samples of organic of healthy papayas fruits collected from production orchards in the metropolitan area of Palmas city in Tocantins. The process of isolation follows the methodology of Janisiewicz et al. (2010) with modifications. The fruit samples were washed in polyethylene bags with saline solution (0.85%). The washings were discarded, and fresh solution was added to the polyethylene bags. The fruit was washed again using a sonication bath (Ultra cleaner 1600 A, UNIQUE) for 1 min. An aliquot of 100 µl was collected and transferred to Petri dishes with nutrient yeast dextrose agar - NYDA (0.3% extract de carne; 0.5% yeast extract; 0.5% peptone; 1% glycose; 2% agar; 0.02% chloramphenicol).

The pathogen *C. gloeosporioides* (UFT 9500) was obtained from Carlos Augusto Rosa microorganisms culture collection from the Federal University of Tocantins. The microorganism was reactivated through its cultivation on Potato Dextrose Agar (PDA) and incubation for 7 days at 25 ° C.

Growth temperature was determined for each strain at 37°C and the ability of yeasts to inhibit pathogen growth was tested on NYDA agar as described by Spadaro et al. (2002). Briefly, a line of the antagonist was placed the of it approximately 25 mm from the border. A 5 mm mycelial disk of the pathogen was placed 30 mm from the border and 30 mm from the antagonist. Plates were incubated at 25ºC until the mycelium of the pathogen reached the Petri dish border opposite the antagonist strip. The radial growth of the pathogen towards the antagonist was then measured. Experiments were repeated three times.

The yeasts isolated and selected in the previous tests were evaluated as biocontrol agents against *C. gloeosporioides* in papaya wounds. Organic papayas were purchased from a local commercial orchard and selected for lack of injury. The fruits were disinfected to the surface with sodium hypochlorite (1%) for 3 min and then washed with sterile water and dried in a sterile chamber. Three wounds (5 mm wide x 2 mm deep) were cut at the equatorial region of each fruits. Wounds were inoculated with 20 µl of a yeast suspension (10^8 cells/ mL^{-1}). After 20 min the wounds were inoculated with 20 µl of suspension of pathogenic conidia (2 x 10^5 conidia/ mL^{-1}). The integrated biocontrol tests were performed with fruits inoculating the yeast in combination with 1% sodium bicarbonate and pathogen. 20 µl aliquots of yeast suspension were deposited on the papaya wound immediately after the wound, then allowed to dry and it was inoculating 20 µl of 1% sodium bicarbonate

solution allowed to dry and was applied 20 µl of pathogen suspension. The inoculated fruits were incubated in 100% humidity chamber at 25 ° C for 10 days. Controls included inoculation with single pathogen, GY broth with only yeast and 1% sodium bicarbonate. For each test, ten fruits were arranged randomly in blocks for treatment. After the incubation period, the wounds were examined and the wounds diameters were measured. The experiments were repeated three times.

The percentage of disease severity reduction (DSR %) was calculated by the equation: DSR (%) = [(DSc - DSt)/DSc] X 100, in which DSc = average area with lesions on the positive control and DSt = area with lesions on the treated fruits (ASSIS et al., 1999). Only the mechanically wounded region of the papaya was used for the assessment of disease reduction. The experiments of the disease severity reduction had been analyzed by the test of Tukey with 5% confidence.

The molecular identification was made only for the isolate selected in in vivo tests. The species was elucidated by sequencing the D1/D2 genic regions in both directions. according to Kurtzman and Fell (1998) and using the API system. To find sequence similarity there were searches performed with BLAST network service of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

A total of 85 isolated yeasts were obtained and tested according to characteristics of production of antagonistic substances and growth at 37ºC. With respect to the production of antagonistic substances against fungus *C. gloeosporioides* the results showed that 97.6% of the isolates did not showed phytopathogen inhibition rates higher than 50%, which is necessary to be considered positive. However, the UFT 5845 isolate had the highest inhibition index with 54.9%. Another isolate, UFT 5866 inhibited growth by 34.3%. Even though it did not reach the 50% rate necessary to be considered positive, this isolate showed a possible potential for the production of diffusible substances in the culture medium and was able to cause antibiosis effect to the fungus, therefore, was considered positive.

The antibiosis effect generated by microorganisms, as found in this study may be due to the production of various compounds, such as antibiotics, siderophores or toxic metabolites (VARGAS et al., 2012). In this study only, the isolates that showed negative results were used in subsequent tests, thus minimizing the possibility of some isolated produce harmful substances to human health. For this reason, the analysis of the toxicological profile of these compounds becomes indispensable and must be rigorously investigated, considering the possibility that these agents leave a toxic residue in fruits *in natura* (JANISIEWICZ; KORSTEN, 2002; KUPPER et al., 2013). Thus, the 83 isolates which presented negative results in the antagonism test were used to verify the ability to grow at a temperature of 37ºC. Among these, only four isolates (UFT 5822, UFT 5852, 5859 UFT, UFT 5872) were not able to grow at the tested temperature (37°C), for this reason they were selected for the next stage of the study. The absence of growth at temperatures at or above 37 °C is an important factor considering the selection of a satisfactory biocontrol agent, because this absence of growth reduces the chances of colonization in humans, which decreases the possibility of selecting a pathogenic yeast (PIMENTA et al., 2010).

The isolated UFT 5852, which was selected in the tests mentioned previously, was identified by molecular analysis. In which the consensus sequence D1/D2 exhibited ≥99% homology with the sequence deposited in GenBank for the corresponding species *Anthracocystis grodzinskae*. The phylogenetic tree, which was derived from the analysis of 26S rDNA in the domain D1/D2, is shown in Fig. 1 and it has presented the same relationship between the isolate and the species. The obtained sequence was deposited in GenBank under the following accession KX588719. After the molecular identification of the species, bibliographic searches were performed for the survey of previous reports of pathogenicity of the isolate for humans other animals, this search resulted in the absence of reports (PIATEK et al., 2015).

In this study, the results showed that the yeast *A. grodzinskae*, by itself, reduced the severity of disease caused by *C. gloeosporioides* in 93.7% in comparison to the positive control that had 100% incidence of the disease after 7 days of incubation. However, when using sodium bicarbonate (1%) against the phytopathogen, it showed a decrease of the disease severity in 100%, which was expected based on preliminary studies.

By integrating the control performed by the yeast *A. grodzinskae* with 1% sodium bicarbonate, the result showed a decrease of 84.4%. However, statistically, the treatments did not presented significantly difference at 5% significance by Tukey test, which highlights the positive result in the disease control due to the use of the yeast *A. grodzinskae* as also the use of the GRAS substance with the intercropping of the two treatments. The average of the lesions was also statistically equal to the 5% level of significance by Tukey test, differing only from the positive control (Table 1).

Several previous studies have shown that sodium bicarbonate has played an important role in the control of postharvest diseases, for example, the work of Geng et al. (2011), who had obtained similar results to this study by combining the yeast *Kluyveromyces marxianus* and baking soda to control green mold in citrus, achieving a reduction of 70% about the disease incidence when used only yeast and approximately 58% by integrating the treatment of this yeast with sodium bicarbonate.

Gamagae et al. (2003) however used 2% sodium bicarbonate solution and the yeast *Candida oleophila* to control Anthracnose in papaya during the storage. Treatment

with 2% sodium bicarbonate did not significantly affect the growth of the biocontrol agent. However, control of anthracnose increased when combined with *C. oleophila* strain resulting in reduction around 50% of disease.

The lesions that inevitably occur during harvesting, transportation and handling, can not only damage the fruit, but also serve as a gateway to the invasion of microorganisms, especially for primary pathogens (JANISIEWICZ; KORSTEN, 2002). Among the mechanisms of action, the competition for nutrients and/or space is considered a key factor in which the antagonists suppress post-harvest pathogens, for this reason a good biocontrol agent should present the ability to rapidly grow and colonize the fruit (PIMENTA et al., 2010).

Considering this, the treatments with *A. grodzinskae* and baking soda kept the lesions of the fruits protected by the period that lasted the experiment (7 days), without showing characteristic symptoms of the disease. It was also observed that the application of the yeast and baking soda, alone or in combination, did not show any injury in the fruit, which is an important characteristic for the establishment of a disease control strategy.

The use of antagonistic yeasts has been considered as a possible strategy to substitute the use of synthetic fungicides, several studies have pointed that the use of microorganisms in postharvest protection (SPADARO; DROBY, 2016). However, most of these studies have been concentrated to a small group of fruits such as apple, pear, strawberry and citrus (LIMA et al., 2013), with only a few papers describing the use of biocontrol agents in papaya and the activity of these agents against the fungus *C. gloeosporioides*.

Thus, it is necessary to intensify researches directed to this fruit, considering that the post-harvest losses generated by this phytopathogen reach large proportions in the global and regional economy and that the use of pesticides is still the primary way for controlling this fungus.

The assay of integrated biological control surprisingly found that the use of 1% sodium bicarbonate alone was the most effective strategy for the control of the phytopathogen *C. gloeosporioides*. This fact is interesting because it is an inexpensive and easy methodology to the producers, since the sodium bicarbonate is a food additive that has no phytotoxicity, has low cost, good availability in the market, easily storage, handling and application (ZHOU et al., 2015). In addition, its inhibitory activity may be related to pH, since the sodium bicarbonate can change the pH of the microorganisms growing environment and therefore affect the survival of the microbial cells (ZHOU et al., 2015).

Thus, the application of a 1% sodium bicarbonate solution satisfactorily controlled the development of Anthracnose in papaya inoculated with *C. gloeosporioides*, thus being an effective, accessible and low-cost strategy. The yeast *A. grodzinskae* was able to control the anthracnose in papaya, even when inoculated without additives. However, further studies are needed to elucidate the mechanisms of action of sodium bicarbonate on the phytopathogenic fungi of *C. gloeosporioides*.

Table 1. Assay of integrated biocontrol test *in vivo* of the fungus *C. gloeosporioides* after 7 days of incubation.

Treatments	Lesion diameter (mm)	Disease reduction severity (%)
Control *C. gloeosporioides* (+)	19.3[a]	0.0[b]
Control water (-)	-	-
Control yeast (-)	-	-
Control sodium bicarbonate (SB) (-)	-	-
Yeast +SB (-)	-	-
C. gloeosporioides + Yeast	1.2[b]	93.7[a]
C. gloeosporioides + SB (1%)	0.0[b]	100[a]
C. gloeosporioides + yeast + SB (1%)	3.0[b]	84.4[a]

* Averages followed of different lowercase letters in the columns, which differ from each other by Tukey test ($p \leq 0.05$).

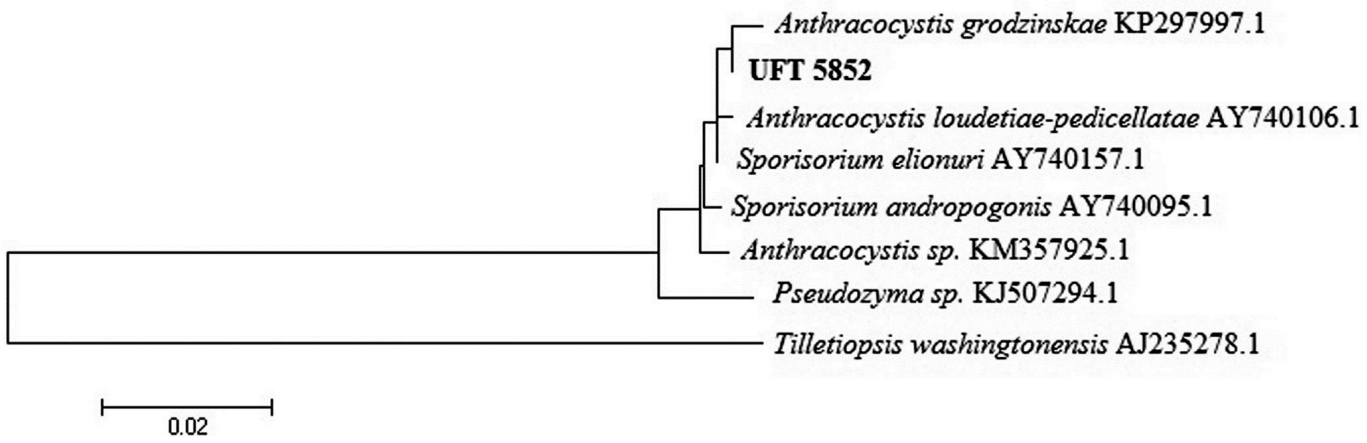

Figure 1. Phylogenetic Tree demonstrating the relationship between the isolate UFT 5852 and the species Anthracocystis grodzinskae, Tilletiopsis washingtonensis was used as outgroup in the analysis. And the bootstrap consensus was inferred from 1000 replicates, which were derived from the analysis of 26S rDNA in the domain D1/D2. The evolutionary distances were calculated using the Jukes-Cantor method.

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References

ASSIS, S.M.P.; MARIANO, R.L.R. Antagonism of yeasts to *Xanthomonas campestris pv.* Campestris on cabbage phylloplane in field. **Revista de Microbiologia**, São Paulo, v.30, p.191-195, 1999.

CAI, Z.; YANG, R.; XIAO, H.; QIN, X.; SI. Effect of preharvest application of *Hanseniaspora uvarum* on postharvest diseases in strawberries. **Postharvest Biology and Technology**, Leuven, v.100, p.52–58, 2015.

GAMAGAE, S.U.; SIVAKUMAR, A.D.; WILSON W.R.S.; WIJESUNDERA, R.L.C. Use of sodium bicarbonate and *Candida oleophila* to control anthracnose in papaya during storage. **Crop Protection**, Heidelberg, v.22, p.775–779, 2003.

GENG, P.; CHEN, S.; HU, M.; RIZWAN-UL-HAQ, M.; FEI QU, K.L.; ZHANG, L. Combination of *Kluyveromyces marxianus* and sodium bicarbonate for controlling green mold of citrus fruit**. International Journal of Food Microbiology**, Grugliasco, v.151, p.190–194, 2011.

GUPTA, R. K.; CHOUDHARY, B.; NAGPURE, A. Biological control of toxigenic citrus and papaya-rotting fungi by *Streptomyces violascens* MT7 and its extracellularmetabolites**. Journal of Basic Microbiology,** Berlin, v.55, p.1343-1356, 2015.

JANISIEWICZ, W.J.; KORSTEN, L. Biological control of postharvest disease of fruits. University of Pretoria, Pretoria. **Phytopathology**, St. Paul, v.40, p. 411-441, 2002.

JANISIEWICZ, W.J.; KURTZMAN, C.P.; BUYER, J.S. Yeasts associated with nectarines and their potential for biological control of brown rot. **Yeast**, New Jersey, v.27, p.389-398, 2010.

JANISIEWICZ, W.J.; PIMENTA, R.S.; JURICK II, W.M. A novel method for selecting antagonists postharvest fruit decays originating from latent infections. **Biological Control**, Dordrecht, , v.59, p.384-389, 2011.

KUPPER, K.C.; CERVANTES, A.L.L.; KLEIN,M.N.; SILVA, A. C. Avaliação De Microrganismos Antagônicos, *Saccharomyces cerevisiae* e *Bacillus subtilis* para o controle de *Penicillium digitatum*. **Revista Brasileira de Fruticultura**, Jaboticabal, v.35, n.2, p.425-436, 2013.

KURTZMAN, C.P.; FELL, J.W. **The yeasts. A taxonomic study**. Amsterdam: Elsevier, 1998.

LI, X.; ZHU, X.; ZHAO, N.; FU, D.; LI, J.; CHEN, W.; CHEN, W. Effects of hot water treatment on anthracnose disease in papaya fruit and its possible mechanism. **Postharvest Biology and Technology**, Leuven, v.86, p.437- 446, 2013.

LIMA, J.R.; GODIM, D. M.F.; OLIVEIRA, J.T.A.; OLIVEIRA, F.S.A.; GONÇALVES, L.R.B.; VIANA, F.M.P. Use of killer yeast in the management of postharvest papaya anthracnose. **Postharvest Biology and Technology**, Leuven, v.83, p.58–64, 2013.

PALOU, L.; ALIB, A.; FALLIKC, E.; ROMANAZZID, G. GRAS, plant- and animal-derived compounds as alternatives to conventional fungicides for the control of postharvest diseases of fresh horticultural produce. **Postharvest Biology and Technology**, Leuven, v.122, p.41–52, 2016.

PIĄTEK, M.; MATTHIAS, L.; NOUROU, S. Y. A molecular phylogenetic framework for *Anthracocystis (Ustilaginales)*, including five new combinations (inter alia for the asexual *Pseudozyma flocculosa*), and description of *Anthracocystis grodzinskae* sp. nov. **Mycological Progress**, Heidelberg, v.14, p.88, 2015.

PIMENTA, R.S.; SILVA, J. F.M.; COELHO, C.M.; MORAIS, P. B. Integrated control of *Penicillium digitatum* by the predacious yeast *Saccharomycopsis crataegensis* and sodium bicarbonate on oranges. **Brazilian Journal of Microbiology**, São Paulo, v.41, p.404-410, 2010.

SPADARO, D.; DROBY, S. Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists, **Trends Food Science & Technology**, Norwich, v.47, p.39-49, 2016.

SPADARO, D.; VOLA, R., PIANO, S.; AND GULLINO, M.L. Mechanisms of action and efficacy of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens on apples. **Postharvest Biology and Technology**, Leuven, v.24, p.123-134, 2002.

VALENZUELA, N.L.; ANGEL, D.N.; ORTIZ, D.T.; ROSAS, R. A.; GARCIA, C.F.; SANTOS, M. O. Biological control of anthracnose by postharvest application of *Trichoderma spp*. on maradol papaya fruit. **Biological Control**, Dordrecht, v.91, p.88-93, 2015.

VARGAS, M.; GARRIDO, F.; ZAPATA, N.; TAPIA, M. Isolation And Selection Of Epiphytic Yeast For Biocontrol Of Botrytis cinerea Pers. On Table Grapes. **Chilean Journal of Agricultural Research**, Chillán, v.3, p.332-337, 2012.

VERO, S.; MONDINO, P.; BURGUEÑO, J.; SOUBES, M. Characterization of biocontrol activity of two yeast strains from Uruguay against blue mold of apple. **Postharvest Biology and Technology**, Leuven, v. 26, p. 91-98, 2002.

ZHOU, J.; LAI, T.; BAI, X.; WANG, Y.; SHI, N.; ZHOU, T. Inhibitory effect of exogenous sodium bicarbonate on development and pathogenicity of postharvest disease *Penicillium expansum*. **Scientia Horticuturae**, New York, v.187, p.108-114, 2015.