Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 7, No. 5, p. 50-58, 2015
http://www.innspub.net

INNSPUB

RESEARCH PAPER

OPEN ACCESS

Evaluation of antifungal activity of allyl isothiocyanate against *Rhizopus stolonifer*

Farborz Zare Nahandi*, Saeedeh Alizadeh-Salteh

Department of Horticultural Science, University of Tabriz, Tabriz, Iran

Article published on November 11, 2015

Key words: Allyl isothiocyanate, *Rhizopus stolonifer*, Antifungal activity, Mycelia growth.

Abstract

Antimicrobial volatile substances from plants have become known as a suitable alternative to synthetic fungicides and food preservatives.Because of health risks for mankind and environment of chemical fungicides, nowadaysthe use of biological alternatives is growing fast.In this study, the antifungal activity of purified Allyl isothiocyanate from cruciferous plants was evaluated against*Rhizopus stolonifer* isolated from peach. Conidia germination and mycelial growth of *R. stolonifer*was tested at different concentrations. The results showed a significant antifungal activity of AITC and could inhibit spore germination and mycelium growth completely at 0.64 for mycelia (89.04%) and 0.24 microliter per plate for conidia germination (100%). It was concluded that the natural compound examined in the present study could be used as antifungal agents against food spoilage and mycotoxin producing fungi. Further studies on their effect on other important fungal species and also their anti-fungal activity are required.

***Corresponding Author:** Farborz Zare Nahandi ✉ fzaare@gmail.com

Introduction

Improvement of nutrition of people is one of the most essential issues nowadays. This depends on consumption of high quality raw materials that used for processing of food. They should not be contaminated by chemicals or propagules of microorganisms (Samson *et al.*, 1988). Fruits and vegetables are grown in various types of soil, applying different agro-technical and agrochemical means. Some conditions promote the development of various groups of microorganisms that contaminate food and feedstuff that can find in places of their growth, ripening, and while harvesting (Carlile and Wathinson, 1996).

Microorganisms of some species, especially fungi, intensively developing on food, fruit and vegetables, and leads to heavily contamination, thus making unsuitable for consumption and high value of economic loses (Frazier and Westhoff, 1988). One of the high distributed micromycetes, is Rhizopus that can produce toxic metabolites, like compounds of the aflatoxins group (Samson and van Reenen-Hoekstra, 1988). Rhizopus is a genus of common saprophytic fungi on plants and specialized parasites on animals. They are found on a wide variety of foodstuffs, including "mature fruits and vegetables (Kirk *et al.*, 2008). Some Rhizopus species can cause fungal infection on human like zygomycosis and can be fatal. Rhizopus infections may also be a complication of diabetic ketoacidosis (Chinn and Diamond, 1982). Rhizopus is one of the Mucorales most commonly associated with infection in humans, which can develop in diabetic or immunocompromised patients (Warnock, 1995). Within the genus rhizopus, the formation of anticancer drugs rhizoxins and toxic rhizonins have been described (Jennessen, 2005). Although, the production of rhizonin is not caused by the fungus, but symbiont bacteria of the genus *Burkholderia* that localized in the fungal cytosol (Lackner *et al.*, 2009).*Rhizopus stolonifer* spores are usually dispersed in hot dry weather and they contain allergic proteins, which can cause respiratory and nasal symptoms in humans, such as coughing, chest discomfort and allergic reactions (Zhang *et al.*, 2005).On the other hand, *Rhizopus stolonifer* can cause rhizopus soft rot on many of fruits, especially on peaches, plums, other stone fruits, bananasthat leads to high loses of yield and decreases export of them. There are some chemical fungicides that have been used to control of Rhizopus. The widespread use of fungicides has significant drawbacks including increased cost, handling hazards, chemical residues on food, and threat to human health and environment (Paster and Bullerman, 1988), so there is increased interest in finding safer alternatives like natural plant protectants like plant hormones (salicylic acid) (Panahirad*et al.*, 2012) and essential oils (Alizadeh-Salteh *et al.*, 2010) as to replace synthetic chemical fungicides and have low mammalian toxicity, less environmental effects and wide public acceptance (Hamilton-Kemp *et al.*, 2000).

Plant extracts have demonstrated antimicrobial effects mediated by several compounds, such as phenolics, flavonoids, allicin, thiosulfinates, betalain and phytoalexins (Harris *et al.,* 2001), and there is an ongoing and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action (Parekh and Chanda, 2007).

In addition of essential oils, there are another group of compounds includes some molecules that come from the enzymatic hydrolysis of glucosinolates (GLs) such as isothiocyanates (ITCs) that can be used as 'biofumigants'. Isothiocyanates (ITCs; R–N5C5S) are the products of the reaction of plant glucosinolates with myrosinase, an enzyme released by the disruption of plant tissues. This myrosinase–glucosinolate system is present in plants of the family Brassicaceae, such as cauliflower, broccoli, mustard and cabbage. ITCs are volatile substances that display an inhibitory effect on a variety of pathogenic microorganisms at low concentrations, making them promising antimicrobial candidates. The use of allyl ITC (AITC) is already approved in Japan for food preservation, provided it has a natural plant source

(Nadarajah *et al.*, 2005).Allyl isothiocyanate (AITC), C_4H_5NS, a natural compound commonly found in the Brassicaceae family (Tookey *et al.,* 1980), possesses a strong antimicrobial activity against bacteria and other pathogenic bacteria (Inoue *et al.*, 1983). The major pungent component of black mustard (*Brassica nigra*) and brown mustard (*B. juncea*), which is the same as that of wasabi (*Eutrema wasabi* Maxim.), is allyl isothiocyanate (AIT). AIT is released from a naturally occurring glucosinolate, sinigrin, by the action of myrosinase (Fig. 1). It has been used as a preservative compound and is considered safe for human consumption (Kermanshai *et al.*, 2001).

Studies have shown that allyl isothiocyanate (AIT), has antioxidant and antimicrobial properties that inhibit a variety of pathogens at low concentrations (Nadarajah*et al.*, 2005). In Japan, the use of AIT from natural sources is allowed, and it is classified as safe by the Food and Drug Administration (FDA) of the United States (Wang, Chen& , Yin, 2010).It has been demonstrated that AITC effectively inhibits a variety of pathogenic microorganisms such as *M. laxa*, *R. stolonifer*, *Neofabra alba*, *B. cinereae* (Mari *et al.,* 2002). It has been used as a preservative compound and is considered safe for human consumption and believed to be conductive to health (Shin *et al.*, 2004; Troncoso *et al.*, 2005).

Despite the fact that Allyl isothiocyanate is a common and important compound which is present in large amounts in Brassicaceae, no comprehensive study has been conducted to investigate the inhibitory effect of this substance on the growth and development of *Rhizopus* rot. Therefore, in this study,it wastrying to evaluate the efficacy of this compound for controlling this pathogenic fungus in peach fruits.

Material and methods

Isolation, purification and identification of Rhizopus stoloniferfrom peach

Peaches with obvious Rhizopus sporulation, used for isolation of *Rhizopus stolonifer*. Contaminated samples were surface-sterilized with 5% sodium hypochlorite solution for one minute before being rinsed three times with sterilized, distilled water, then shacked overnight. After 3 days and filtration by cheesecloth, fungus hypha were subcultured on PDA (potato dextrose agar) containing antibacterial to obtain pure cultures. The Petri dishes were incubated for 7 days at 25°C and observed daily for the emergence of colonies. Isolates were purified through single-spore methods and then transferred to PDA slants. Species were determined using microscopic features.Allyl isothiocyanatewith at least a nominal purity of 95%, were purchased from Sigma–Aldrich.

Fungal spore suspension and inoculums preparation

To obtain spores of *Rhizopus stolonifer* inoculum, at first we sub-cultured the plate of the fungus by streaking the spores onto fresh potato dextrose agar (PDA) plates and incubated for 7 d at 25 °C. The seven days old spore cultures of the fungus was suspended in sterile distilled water containing 0.001 ml L^{-1} of Tween 80. The spore suspensions were then filtered with 6 layers of cheesecloth to remove debris such as mycelia and condensed agar fragments, and its concentration was adjusted to 10^3 and 10^6 fungal spore ml^{-1} suspensions using a haemocytometer for conidia germination and mycelia growth respectively. This suspension was used to study the effect of AITC on mycelia growth and conidia germination of *R. stolonifer*.

Control of Rhizopus stolonifer by AITC

The effect of AITC on inhibition of conidia germination and mycelium growth of *R. stolonifer* was tested *in vitro* on malt extract agar in 6 concentrations.

Effects of AITC on conidia germination

The inhibition of conidia germination was tested by spreading 100µl of *R. stolonifer*conidia suspension (10^3 conidia ml^{-1}) on Petri dishes containing 20 mL of malt extract agar (MEA). In each case different aliquots of pure AITC (0, 0.01, 0.03, 0.06, 0.12 and 0.24 µ/l) were placedusing a microsyringe, on a Paper filter (Whatman No. 1), positioned inside the cover of

petri dishes with 90 mm diameter. The dishes were quickly closed and sealed with Parafilm and incubated at 20 °C. The dishes were opened after 3 days for conidia germination and the paper filter removed to evaluate fungicidal activity of AITC by allowing the fungus to grow for the next 7 days, respectively. Petri dishes inoculated with *R. stolonifer* but treated with distilled water used as a control. Conidia germination was determined by observing the conidia directly with a light microscope; at least 500 conidia for each treatment and control.

In a second set of experiments, the appropriate exposure time to AITC vapours was evaluated only for conidia germination. AITC was added to the paper filter and the Petri dishes immediately sealed with Parafilm and kept at 20 °C. The AITC concentrations were fixed at ED50 values. After 1.5, 3, 6, 12 and 24 h the dishes were opened and the filter paper removed. Three days after opening, germinated conidia were evaluated as noted above. Dishes sealed with Parafilm for 24 h, without any AITC addition, were the control references. AITC exposure time was represented by five dishes and the experiment was done twice.

Effects of AITC on mycelia growth

Mycelium growth inhibition was evaluated by placing a plug (6 mm diameter) from an actively growing culture (the margins of a 7-day-old culture) in the center of a MEA plate.

In each case different aliquots of pure AITC (0, 0.04, 0.08, 0.16, 0.32 and 0.64 µl/plate) were placed, using a microsyringe, on Paper filter (Whatman No. 1), positioned inside the coverof petridishes with a 90 mm diameter. The dishes were quickly closed and sealed with Parafilm and incubated at 20 °C. The dishes were opened after 7 days for mycelium growth. Petri dishes inoculated with *R. stolonifer*but treated with distilled water used as a control. Mycelium growth was measured with a centimetre.

Data were expressed as percent of conidia germination or mycelium growth inhibition compared with the control. In turn, parafilm and filter papers were removed from the dishes and another assessment was carried out after 7 days at 20°C in order to evaluate whether the activity of the compounds was fungistatic or fungicidal. The experiments were performed twice.

When mycelial growth was asymmetrical, four diameter measurements were determined and averaged. Inhibition of mycelial growth (IG) was calculated as a percentage from the difference between the growths of treated and control mycelium, as shown below:

GI (%) = 100 (C-T) / C

Where C is mycelium diameter in control dishes and T ismycelium diameter in treated dishes.

Statistical analysis

The statistical effect of the AITC concentrations on *R. Stolonifer* growth was studied by analysis of variance based on a completely randomized design. The means comparisons were made using Duncan's multiple range tests at P≤0.05. All statistical analyses were carried out using the SPSS software.

The EC50 and EC95 values were determined by the linear regression of the probit of the test fungus percentage inhibition and the log of the studied compound concentrations using the STATGRAPHICS software.

Results

Growth inhibition effect of AITC against spores of R. stolonifer (GI %)

The results of analysis of variance showed that different concentrations of AITC (0, 0.04, 0.06, 0.10, 0.12 and 0.24 µl/plate) had significant differences on growth inhibition of spores of *R. stolonifer* (Fig. 2).

Obtained results showed that AITC at 0.24 µl/plate completely controlled spore germination of fungus (100%). While, AITC at 0.04 and 0.06 µl/plate had a negative effect on fungus spore germination control

and in fact lead to increase in spore germination of *R. stolonifer* (GI: Respectively -5.47 and -3.69 %) (Fig. 3). Applying 0.10 AITC µl/plate didn't have susceptible growth inhibition (7.78%). On the other hand, use of AITC at 0.12 µl/plate lead to relatively high growth inhibition of spore germination of *R. stolonifer* (64.21%) (Fig. 4).

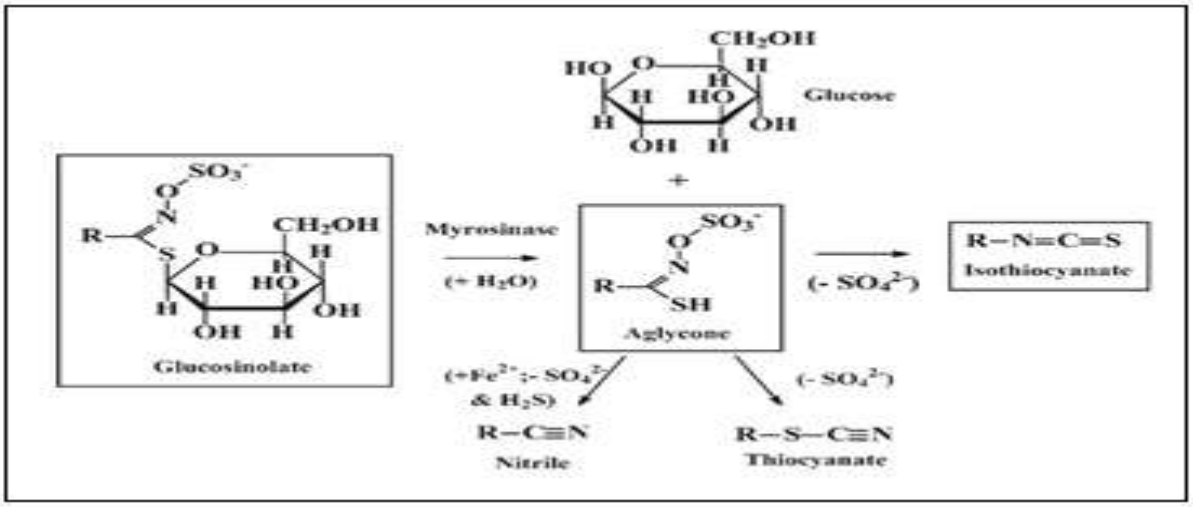

Fig. 1. Hydrolysis of glucosinolates by myrosinase and formation of isothiocyanates.

The effect of AITC on mycelia growth of R. stolonifer (GI %)

The results obtained from Analysis of variance of different concentration of AITC (0, 0.04, 0.08, 0.16, 0.32 and 0.64 µl/plate) showed significant effect on growth inhibition of mycelia growth of *R. stolonifer* (Figs. 5 and 6).

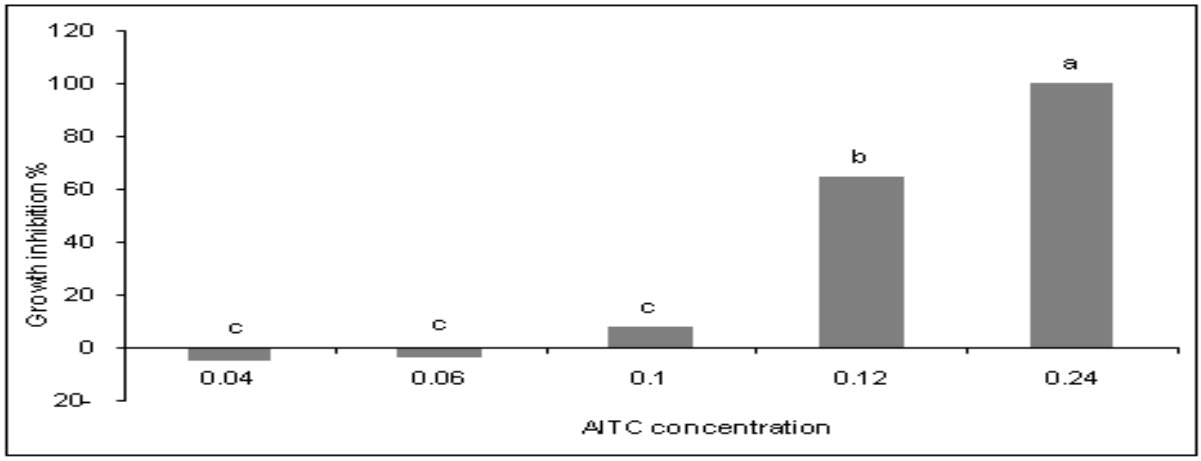

Fig. 2. The effect of different Concentrations of AITC against spore germination of *R. stolonifer*.

On the basis of Growth inhibitory percent of different concentrations of AITC in control of mycelia growth of *R. stolonifer*, using 0.64 µl/plate lead to highest control of mycelia growth (89.04%) (Fig. 7). AITC at 0.04 and 0.08 µl/plate showed the lowest inhibition growth (respectively 2.55 and 4.32 %) (Fig. 7). Applying of AITC at 0.16 and 0.32 µl/plate showed respectively 16.86 and 52.57 % mycelium growth inhibition of *R. stolonifer*.

Discussion

Fungal decay is one of the major causes of rapid post-

harvest deterioration of fresh produces and can be Threatening agent for human health. The most widely used methods to limit fungal decay is the application of fungicidal substances. However, due to public concerns on health risk and environmental contamination, alternative methods are needed. Investigations on the beneficial use of nonchemical techniques for minimizing our dependency on potentially hazardous chemicals to reduce decay and post-harvest losses of fruits and vegetables have gained growing interest. Several naturally occurring essential oils of plants like Isothiocyanates have been reported to have antimicrobial properties and have shown promise in reducing post-harvest diseases and disorders in horticultural crops (Wang *et al.*, 2008; Wang *et al.*, 2010).

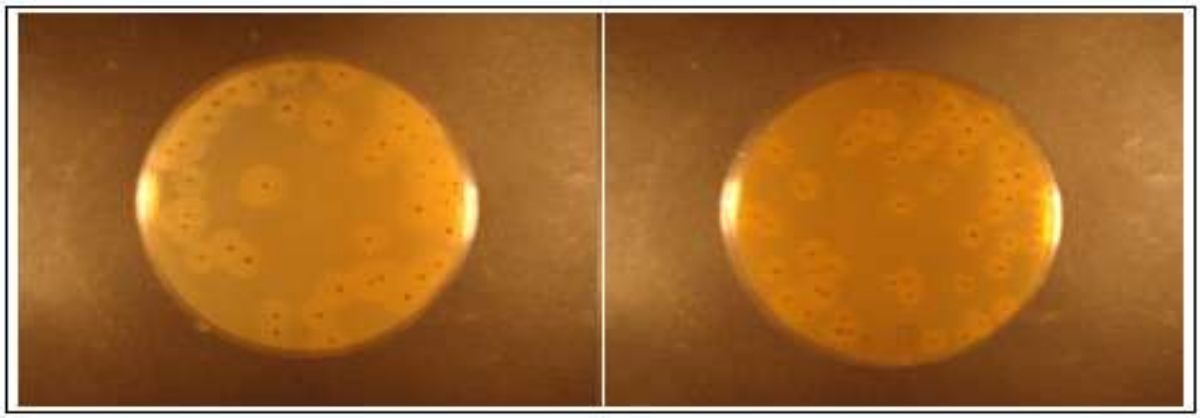

Fig. 3. The effect of AITC on conidia germination of *R. stolonifer*: Left: control, right: 0.04 µl/plate AITC.

In the present study, we have shown that AITC has a significant inhibitory effect on condo germination and mycelia growth of *Rhizopus stolonifer*, one of the important micromycetes that produce mycotoxines and leads to postharvest deacades of food and feedstuff and can be dangerous for human health.

Our results are in agreement with those published by Mari *et al.*, 2008 that mentioned potent inhibitory activity of AITC against *Monilinia laxa* and results of Neri *et al.*, 2005in control of *P. expansum*.

Antimicrobial activity of AITC is well documented in the literature, and it can kill fungal propagules by vapor action, thus acting as a fumigant (Lin *et al.*, 2000; Mari *et al.*, 2002). The antimicrobial activity of AITC is suggested to involve a reaction with thiol groups of glutathione or redox-active proteins, with subsequent inhibition of sulfhydryl enzyme activities and inhibition of redox-based defenses (Jacob and Anwar, 2008). The activity of AITC varies with the structure of the molecule, but variations are also noticed amongst identical bacterial species for one AITC.

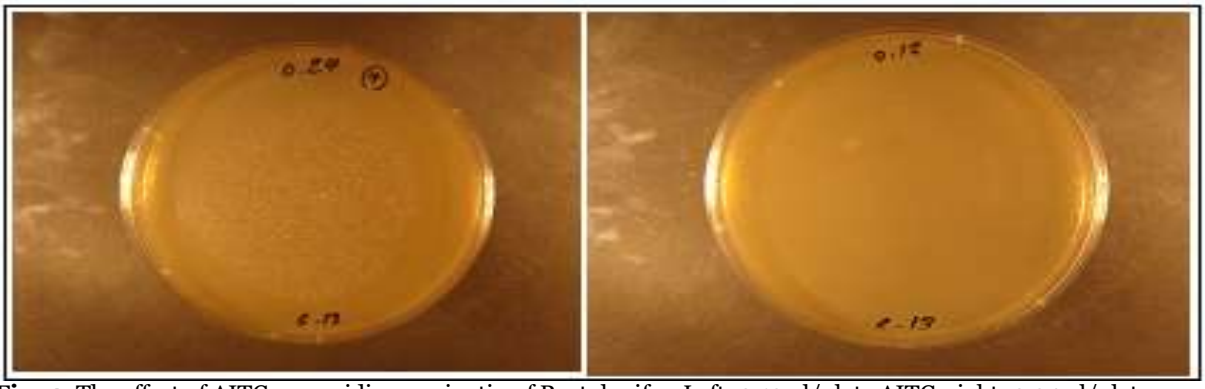

Fig. 4. The effect of AITC on conidia germinatin of R. stolonifer. Left: 0.12 µl/plate AITC, right: 0.24 µl/plate.

Therefore, we can postulate that the efficacy of the ITC may depend on both the rate of spontaneous degradation of AITC-thiol conjugates and of the detoxification mechanisms of the bacterial isolate.

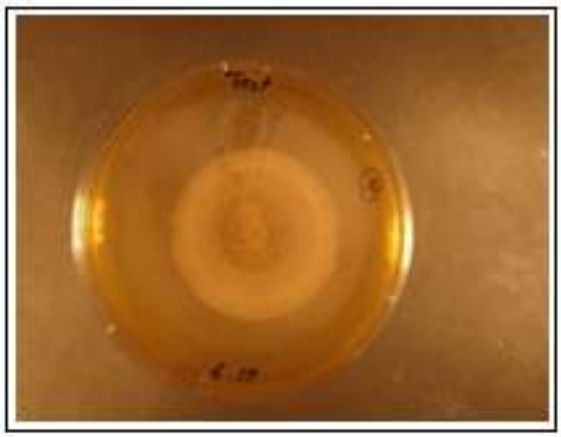

Fig. 5. Mycelia growth of *R. stolonifer* (control).

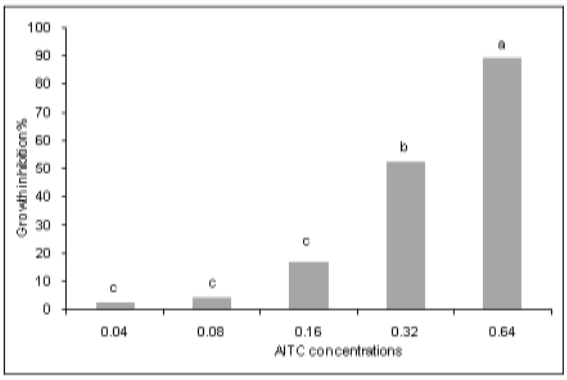

Fig. 6. The effect of different Concentrations of AITC against mycelia growth of *R. stolonifer*.

Additionally, the properties of AITC as antibacterial, anti-fungal and anticancer compounds have increased their interest as food supplements (Singh and Singh, 2012; Zhang, 2012).

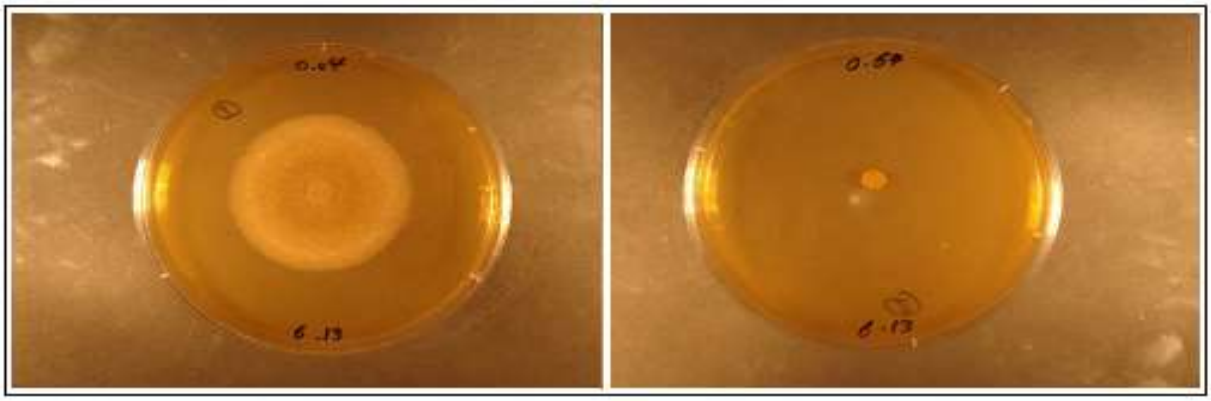

Fig. 7. Disinhibition of mycelia growth of *R. stolonifer* at 0.04µl/plate (left) and completely growth inhibition of mycelia of *R. stolonifer* by using AITC at 0.64 µl/plate (right).

This review intends to summarize the current knowledge about the cellular targets of ITCs, with particular focus on bacterial targets, the potential bacterial resistance to ITCs and the related research perspectives.

Mari *et al* (2002) demonstrated inhibition of conidial germination and mycelial growth of several post-harvest fruit pathogens by BITC and other four natural ITCs. A few years later, Manici *et al* 26 confirmed inhibition of fungal growth of eight plant pathogenic fungi from different taxonomical classes by four ITCs (benzyl, 3-methylsulfinylpropyl, allyl and 2-hydroxy-3-butenyl isothiocyanates).

The mechanism by which ITCs inhibit fungal growth is not yet completely known. Nevertheless, some hypotheses suggest a non-specific and irreversible interaction of the ITC with the sulfidryl groups, disulfide bonds and amino groups of proteins and amino acid residues (Fahey *et al.,* 2001). Reactions with the sulfhydryl group and a decrease in the amount of free amino groups of the proteins' amino acid side residues were observed. The main target of toxic lipophilic compounds on eukaryotic cells is thought to be the cell membrane(sikkema *et al.,* 1995) AITC is a lipophilic compound, therefore it is possible it could react with some enzymes present at the plasma membrane level, causing fungi growth

inhibition or cell death.

Our results showed an interesting antifungal potential of Allyl isothiocyanate against postharvest fruits spoilage fungi. We suggest further research on the efficiency of studied compounds in the prevention of Rhizopus growth on fruits.

Acknowledgement
We would like to thank University of Tabriz for supporting this work under the Research Grant Contract No. 3623-4.

References

Alizadeh-Salteh S, Arzani K, Omidbeigi R, Safaie N. 2010. Essential oils inhibit mycelial growth of *Rhizopus stolonifer*. European Journal of Horticultural Science **75,** 278–282.

Carlile MJ, Wathinson SC. 1996. The Fungi. Academic Press, Horcourt Brace et Company Publishers, London, Boston, San Diego, New York.

Chinn RY, Diamond RD. 1982. Generation of chemotactic factors by *Rhizopus oryzae* in the presence and absence of serum: relationship to hyphal damage mediated by human neutrophils and effects of hyperglycemia and ketoacidosis. Infection and Immunity **38,** 1123–29. Delff., Institute of the Royal Netherland Academy of Arts and Sciences, The Netherlands 1988.

Fahey JW, Zalcmann AT, Talalay P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry **56,** 5–51.

Frazier CW, Westhoff DC. 1988. Food Microbiology. Fourth Edition. Mc Graw-Hill Book Company, New York.

Hamilton-Kemp TR, Archbold DD, Loughrin JH, Andersen RA, McCracken CT, Collins RW. 2000. Stimulation and inhibition of fungal pathogens of plants by natural volatile phytochemicals and their analogs. Current Topics in Phytochemistry **4,** 95–104.

Inoue S, Goi H, Miyauchi K, Muraki S, Ogihara M, Iwatani Y. 1983. Inhibitory effect of volatile constituents of plants on the proliferation of bacteria– Antibacterial activity of plant volatiles. Journal of Antibacterial and Antifungal Agents **11,** 609–615.

Jacob C, Anwar A. 2008. The chemistry behind redox regulation with a focus on sulphur redox systems. Physiologia Plantarum **133,** 469–48010.

Jennessen J, Nielsen KF, Houbraken J, Lyhne EK, Schnurer J, Frisvad JC, Samson RA. 2005. Secondary metabolite and mycotoxin production by the Rhizopus microsporus group. Journal of Agricultural and Food Chemistry **53,** 1833–1840.

Kermanshai R, McCarry BE, Rosenfeld J, Summers PS, Weretilnyk EA, Sorger G. 2001. Benzyl isothiocyanate is the chief or sole anthelmintic in papaya seed extracts. Phytochemistry **57,** 427–435.

Kirk PM, Cannon PF, Minter DW, Stalpers JA. 2008. Dictionary of the Fungi (10th ed.). Wallingford, UK: CABI. 599.

Lackner G, Möbius N, Scherlach K, Partida-Martinez LP, Winkler R, Schmitt I, Hertweck C. 2009. Global Distribution and Evolution of a Toxinogenic Burkholderia-Rhizopus Symbiosis. Applied Environmental Microbiology **75,** 2982-2986.

Lin CM, Preston JF, Wei CI, Lin CM. 2000. Antibacterial mechanism of allyl isothiocyanate. Journal of Food Protection **63,**727-734.

Mari M, Rossi A, Brigati S, Pratella GC. 2002. Antifungal vapourphase activity of allyl-isothiocyanate against *Penicillium expansum* on pears. Plant Pathology **51,** 231–6.

Nadarajah D, Han JH, Holley RA. 2005. Inactivation of Escherichia coli O157:H7 in packaged ground beef by allyl isothiocyanate. International Journal of Food Microbiology **99,** 269–279.

Neri F, Mari M, Brigati S. 2006. Control of *Penicillium expansum* by plant volatile compounds. Plant Pathology **55,** 100-105.

Panahirad S, Zaare-Nahandi F, Safaralizadeh R, Alizadeh-Salteh S. 2012. Postharvest Control of Rhizopus stolonifer in Peach (*Prunus persica*L. Batsch) Fruits Using Salicylic Acid. Journal of Food Safety **32,** 502-507.

Paster N, Bullerman LB. 1988. Mould spoilage and mycotoxin formation in grains as controlled by physical means. International Journal of Food Microbiology **7,** 257–265.

Samson RA, van Reenen-Hoekstra ES. 1988. Introduction to Foodborne Fungi. Third edition. Centralbureau voor Schimmelcultures, Baar. Delff., Institute of the Royal Netherland Academy of Arts and Sciences, The Netherlands 1988.

Shin IS, Masuda H, Naohide K. 2004. Bactericidal activity of wasabi (*Wasabia japonica*) against Helicobacter pylori. International Journal of Food Microbiology **94,** 255–261.

Sikkema J, de Bont JA, Poolman B. 1995. Mechanisms of membrane toxicity of hydrocarbon. Microbiologica Review **59,** 201-22.

Singh SV, Singh K. 2012. Cancer chemoprevention with dietary isothiocyanates mature for clinical translational research. Carcinogenesis **33,** 1833–1842.

Tookey HL, Van Etten CH, Daxenbichler ME. 1980. Glucosinolates. In I. E. Liener (Ed.), Toxic constituents of plant stuffs. New York: Academic Press 103–142.

Troncoso R, Espinoza C, Sánchez-Estrada A, Tiznado ME, García HS. 2005. Analysis of the isothiocyanates present in cabbage leaves extract and their potential application to control Alternaria rot in bell peppers. Food Research International **38,** 701–708.

Wang CY, Wang SY, Chen CY. 2008. Increasing antioxidant activity and reducing decay of blueberries by essential oils. Journal of Agricultural and Food Chemistry **56,** 3587–3592.

Wang SY, Chen CT, Yin JJ. 2010. Effect of allyl isothiocyanate on antioxidants and fruit decay of blueberries. Food Chemistry **120,** 199–204.

Warnock DW. 1995. Fungal complications of transplantation:diagnosis, treatment and prevention. Journal of Antimicrobial Chemotherapy **36,** 73–90.

Zhang Y, Chen J, Chen Y, Dong J, Wei Q, Lou J. 2005. Environmental mycological study and allergic respiratory disease among tobacco processing workers. Journal of occupational health **47,** 181-187.

Zhang Y. 2012. The molecular basis that unifies the metabolism, cellular uptake and chemopreventive activities of dietary isothiocyanates. Carcinogenesis **33,** 2–9.