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In vitro efficacy of garlic extract to control fungal pathogens of wheat

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***In vitro* studies were carried out to investigate the inhibitory effect of allicin in garlic juice on hyphal growth and spore germination of *Drechslera tritici-repetis*, *Bipolaris sorokiniana* and *Septoria tritici*. The purpose was to investigate the possibility of developing an organic agriculture compatible garlic/allicin-based management strategy for the wheat fungal spotting complex. Allicin in garlic juice inhibited radial colony growth of all three pathogens on agar plates. Spore germination of all three pathogens was inhibited by allicin in garlic juice in seeded agar plates and in conidial suspensions. At high concentrations of allicin (> 80 µg/ml) in garlic juice and pure (synthetic) allicin, conidia lysed. The minimum inhibitory amount of pure synthetic allicin which inhibited spore germination completely ranged between 80 to 120 µg/ml, depending on fungal species. At 10 µg/ml, allicin caused morphological abnormalities in hyphae and conidia of *D. tritici-repentis* and *B. sorokiniana*. The current work also provides novel information regarding the effect of allicin-treated hyphae that were collapsed, damaged or thinner when compared with the control. These results suggest that the use of garlic juice is a promising, effective and environmentally friendly management measure against fungal pathogens that could be used in the production of an organically grown wheat crop.**

Key words: Antifungal activity, growth inhibition, wheat, plant pathogens, allicin, garlic extract.

INTRODUCTION

A large number of leaf blights and seed borne diseases affect wheat grown in every cropping season in the agro-ecological Argentine growing area. Leaf spotting diseases can be caused by one or a combination of leaf spotting pathogens. *Cochliobolus sativus* (S. Ito and Kurib.) Drechsler ex Dastur [anamorph: *B. sorokiniana* (Sacc.) Shoemaker], *Pyrenophora tritici-repentis* Died.) Drechs. [anamorph: *D. tritici-repentis* (Died.) Shoemaker] and *Mycosphaerella graminicola* Fuck. Schroether [anamorph: *S. tritici* Rob ex Desm] are the predominant pathogens associated with foliar blights each year, causing spot blotch, tan spot and leaf spot, respectively. *D. tritici-repentis* can also infect wheat kernels causing red or pink smudge and black point.

Severely infected kernels can result in significant down-grading of seed quality. *B. sorokiniana* causes root and crown rots, head and seedling blights, node cankers and black point or kernel smudge (Torp et al., 2006). These pathogens are carried on or within seeds, and can reduce seed germination and seedling emergence (Duveiller et al., 1998; Özer, 2005; Consolo et al., 2009). Leaf spotting diseases reduce the photosynthetic area of leaves, resulting in reduced grain filling and lower yields.

An integrated approach to disease control, including the use of moderately resistant cultivars, chemicals and appropriate cultural practices is currently recommended for fungal disease management in wheat. However, the level of resistance in commercial wheat cultivars is

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relatively low. The effectiveness of chemical seed-treatments is variable and ephemeral and insufficient to protect the wheat plants throughout the growing season. Subsequent infection resulting from residual inoculum on wheat stubble is difficult to manage with chemical control. The goal remains to integrate all available methods for disease control in order to optimise their benefits and minimise their risks for producers, consumers and the environment in a sustainable crop production system. The obvious pollution problems due to indiscriminate use of synthetic pesticides in Argentina (Dall´Armellina, 2006, 2007; Eco Sitio, 2010) and their effects on non-target organisms have prompted investigations on exploiting pesticides of plant origin.

Natural plant products are important sources of new agro-chemicals for the control of plant diseases (Gulter, 1988). It is known that various natural plant products can reduce populations of foliar pathogens and control diseases development, and then these plant extracts have potentials as environmentally safe alternatives and as components in integrated pest management programs (Browers and Locke, 2004). A number of plant species have been reported to possess natural substances that are toxic to several plant pathogenic fungi (Goussous et al., 2010). Plant extracts have played a significant role in reducing the incidence of seed-borne pathogens and in the improvement of seed quality and emergence of plant seeds in the field (Arya and Perelló, 2010; Rukhsana et al., 2010).

Allium sativum (Garlic) juice extract belongs to such non-traditional treatments and among the natural fungicide substances, it has been found most active against many fungal species (Curtis et al., 2004; Slusarenko et al., 2008). Allicin is the most important biologically active substance of *A. sativum* crude extract; it is formed from its precursor, alliin, by the action of allinase enzyme (Josling, 2003). Pharmacologically, allicin is the most important and the most active substance and it is found in the fresh extract of Allium (Vasile et al., 2012). The mechanism of the action of sulfur compounds towards microorganisms is complex and has not yet been fully explained. It is generally recognised that the antimicrobial action of sulfur compounds depends on their hydrophilic or lipophilic character.

Alice and Rao (1987) observed that *A. sativum* extracts significantly reduced seed infection by *Drechslera* on rice and treated seeds had significantly higher viability. On barley in green house and field experiments, allicin used as elicitor was as effective as fungicide against the leaf spot severity caused by *Bipolaris sorokiniana* (Silva et al., 2001; Rodrigues et al., 2002; Rodrigues and Bach, 2003; Antoniazzi et al., 2008). Garlic extract treatment of wheat seeds significantly reduced the incidence of seed-borne fungi, increased seed germination, the number of healthy seedlings and vigour index (Grozav and Foarce, 2005; Khalaf et al., 2011). Moreover, garlic completely controlled the intensity of *B. sorokiniana* and *Fusarium* spp. after the treatment of wheat seeds (Hassan et al., 2005).

It was noticed that essential oil obtained from the garlic bulbs acts as a plant growth regulator with a significant stimulatory effect on monocots (wheat) as well on dicots (cucumber). Consequently, essential oil enhanced plant height, length of the main root and the dry weight of wheat seedlings (Grozav and Foarce, 2005). Promising *in vitro* effects of garlic in controlling fungal pathogens isolated from cassava, cucumber, jute, tomato, rice and sorghum plants were also reported (Jamal-U-Ddin Hajano et al., 2012; Islam et al., 2001; Masum et al., 2009; Nashwa and Abo-Elyousr, 2012; Okigbo et al., 2009; Ruhul et al., 2009). The purpose of this study was to evaluate comparative antifungal efficacy of garlic extract against the pathogenic fungal species *B. sorokiniana, D. tritici-repentis and S. Tritici* causing leaf spotting complex on wheat. The long term aim of this research was to develop and evaluate new alternative methods for managing wheat fungal pathogens in addition to chemical fungicides.

MATERIALS AND METHODS

Organisms

S. tritici, B. sorokiniana and *D. tritici-repentis* argentinian virulent isolates (provided from the CIDEFI-FCAyF,UNLP culture collection) were used in all the experiments. The Stock cultures of *S. tritici* were grown at 20°C for 5 days on Malt Agar, and those of *B. sorokiniana* were grown at 22°C for 10 days on PDA 2% (Potato Dextrose Agar). *D. tritici-repentis* was grown at 22°C and 12 h light-darkness cycles for 7 days on V8 agar. Spore suspensions of the pathogens were prepared by rubbing on the surface of the cultures with an inoculating loop after the addition of distilled water and filtering through two layers of gauze. Spores were counted in a haemocytometer and the suspension was adjusted to the desired concentration by adding distilled water. Suspensions with 3×10^6 *S. tritici* spores/ml, 3×10^5 *B. sorokiniana* spores/ml and spore suspensions with 10^5 *D. tritici-repentis* spores/ml, respectively were used in all experiments.

Garlic extract

Garlic bulbs were purchased from the supermarket and stored at 4°C in the dark until required. Axillary buds from the composite garlic bulb were peeled and weighed and a domestic juicer (Turmix Fabr. No. 1068; Turmix AG, Jona, Switzerland) was used to extract the juice. The juice was poured into a sterile 50v ml Falcon tube and centrifuged at 5,000 rpm (3000 g) for 10 min to separate the majority of the pulp from the liquid (Megafuge 1.0R; Heraeus Instruments, Osterode, Germany). Floating debris was scooped off the top of the liquid with a spatula and discarded. Filtering under pressure separated the remaining pulp from the pure extract (diaphragm vacuum pump; Vacuubrand GmbH, Wertheim, Germany).

The filtrate was transferred into a second sterile 50 ml Falcon tube and sealed. The concentration of allicin in the garlic extract determined by High-performance liquid chromatography (HPLC) and it was used immediately after appropriate dilution. Dilutions were carried out with deionized water.

Table 1. Inhibition of radial colony growth of *B. sorokiniana* by garlic extract after 7 days.

Treatments	Allicin per disc (µg)	Mean colony diameter (mm)	Inhibition (%)
T0 (control)	0	65.3	0.00[b]
T1	13.3	27.8	57.74[a]
T2	26.5	26.7	59.12[a]
T3	53.0	24.1	63.10[a]

T1 = 20 µl of 1:3 dilution (25%); T2 = 20 µl of 1:1 dilution (50%); T3 = 20 µl undiluted garlic juice (2,640 µg/ml per disc).

Quantitative analysis of allicin in garlic extract by high-performance liquid chromatography (HPLC)

The method used was based on that of Krest and Keusgen (2002). Garlic juice was diluted 1:10 with HPLC-grade water and 1.5 ml of a 0.05 mg/ml solution (in methanol) of butyl-4-hydroxybenzoate (internal standard). To protect the column, this mixture was first filtered through a polyethersulfon membrane (0.2 µm pore size, Steriflip; Millipore) before 20 µl was injected into the HPLC (JASCO system with diode array detector; JASCO Deutschland,Gross-Umstadt, Germany). Using the HPLC software Chrompass, a mixed-gradient elution (solvent A, 30% (v/v) HPLC grade methanol with 0,1% formic acid; solvent B, 100% HPLC grade methanol) was carried out. During elution, spectra were recorded between 200 and 600 nm and for the chromatogram, detection was at 254 nm.

Effect of allicin on radial growth of colonies

A plug (10 mm) of mycelium cut from the freshly growing edge of plate cultures of *B. sorokiniana* or *D. tritici-repentis* with a cork borer was inoculated into the centre of a potato dextrose agar (PDA) plate. A dilution series of garlic juice was prepared and pipetted onto 5 discs of filter paper distributed around each plug of fungal inoculum. The resultant colony diameter after 7 days was measured along two axes and the average diameter of replicate colonies was calculated. The percentage of mycelial growth inhibition (P) at each concentration was calculated using the formula: $P = (C - T) \times 100/C$, where C is the diameter of the control colony and T is the diameter of the treated colony.

Agar plate spore germination test

Fungal spore suspensions (1 ml) of *S. tritici*, *B. sorokiniana* and *D. tritici-repentis* were pipetted into a Falcon tube, mixed with 19 ml agar medium at 40°C and poured immediately into 9 cm diameter Petri plates. This ensured a uniform distribution of fungal spores throughout the agar in the assay. Undiluted garlic extract (20 µl containing ≈ 53 µg allicin) was pipetted immediately after preparation onto a series of five filter-paper discs (5 mm) cut with a hole-punch. Plates were incubated for 7 days at 22°C and the diameters of the inhibition zones surrounding the discs were determined.

Conidial germination and hyphal morphology

Conidial suspensions of *B. sorokiniana*, *S. tritici* and *D. tritici-repentis* were thoroughly mixed with an equal volume of a series of dilutions of pure allicin synthesized as in Gruhlke et al. (2010), giving final allicin concentrations of 0 (control), 10, 20, 40, 60, 80, 100 and 120 µg/ml, respectively. The spore suspensions were pipetted onto clean glass slides in order to determinate the relationship between the amount of allicin and inhibition of spore germination. The slides were incubated at 25°C for 5 days. After 24 h of incubation and during 4 consecutive days, samples were mounted in lactophenol cotton blue and examined microscopically using a microscope Leica DM R at 50- to 200-fold magnification. Morphological changes were observed and photographed using a JVC digital camera (KY-F75U) and Discus software (version 32. Hilgers Company, Königswinter, Germany). At least 100 spores were counted and the percentage germination compared to the control was determined. The minimal concentration which caused complete inhibition of germination was taken to be the minimal inhibitory concentration (MIC).

RESULTS AND DISCUSSION

Radial growth

The effects of various additions of garlic juice on radial growth of colonies of *B. sorokiniana* and *D. tritici-repentis* are shown in Tables 1 and 2. For each concentration of garlic extract, the radial growth of colonies was considerably restricted in comparison with the controls. At an amount of 53 µg per disc, allicin inhibited *B. sorokiniana* radial growth by 63% and growth of *D. tritici-repentis* by 67%. Allicin in garlic juice was equally inhibitory for both fungal species at the three concentrations tested. Inhibition of longitudinal extension of hyphae by garlic extract in *B. sorokiniana* and *D. tritici-repentis* was particularly noticeable. Interestingly, the hyphal tips at the edge of the inhibition zone did not grow on, even after 8 days of further observation (Figures 1 and 2). Moreover, a stromatic-like type of colony was induced by garlic extract in *S. tritici* compared with control which showed a yeast-like type of active growth producing salmon-pink secondary conidia (Figure 3).

Fungicidal activity test

The biological activity of fresh garlic extracts on the fungus tested is shown in Table 3. Under the conditions employed, 20 µl of freshly prepared garlic extract caused a halo of inhibition of *B. sorokiniana* growth in seeded agar of approximately 41.1 mm diameter, which corresponded to approximately 53 µg allicin (Figure 4). The size of the inhibition halo for *S. tritici* and *D. tritici-repenis* was 23.1 and 25.5 mm, respectively (Figures 5 and 6). The

Table 2. Inhibition of radial colony growth of *D. tritici-repentis* after 7 days.

Treatments	Allicin per disc (µg)	Mean colony diameter (mm)	Inhibition (%)
T0 (control)	0	68.2	0.00[b]
T1	13.3	25.6	62.47[a]
T2	26.5	23.7	65.25[a]
T3	53.0	22.3	67.31[a]

T1 = 20 µl undiluted garlic extract (2,640 µg/ml per disc) T2 = 20 µl of 1:1 dilution (50%), T3 = 20 µl of 1:3 dilution (25%).

Table 3. The antifungal activity of garlic extract applied as 20 µl (53 µg allicin) on filter paper discs on *B. sorokiniana*, *S. tritici* and *D. tritici-repentis* conidia-seeded PDA 2% agar plates.

Treatments	Zone of inhibition diameter (mm) after days			
	2	4	6	8
B. sorokiniana + CEG	41.1	41.1	41.1	41.1
B. sorokiniana control	0	0	0	0
S. tritici + CEG	25.5	25.5	25.6	15.6
S. tritici control	0	0	0	0
D. tritici-repentis + CEG	23.1	23.1	23.1	23.1
D. tritici-repentis control	0	0	0	0

Control discs were treated with 20 µl of sterile distilled water only.

antimicrobial activity slowly decreased over a period of 14 days.

Effect of garlic juice on conidial germination

The percentage germination of spores of *B. sorokiniana, S. tritici* and *D. tritici-repentis* after 24 h in the presence of garlic juice was determined by counting the number of germinating spores microscopically. The results are shown in Table 4. Pure synthetic allicin at 20 to 60 µg/ml caused a reduction of more than 50% in the germination of spores of *B. sorokiniana*, *S. tritici* and *D. tritici-repentis*, respectively (Figures 7, 8 and 9). The numbers that germinated decreased with increasing allicin concentration. The small numbers of spores that germinated at high concentrations had not developed into normal hyphae after 5 days. The minimal inhibitory concentration (MIC) value that caused 100% inhibition of conidial germination was 80 µg allicin/ml for *B. sorokiniana*, 120 µg/ml for *S. tritici* and 100 µg/ml for *D. tritici-repentis*, observed after four days of incubation.

Morphological effects of garlic juice on germinating conidia

In controls, germ tubes grew rapidly, were regularly branched, and their tips appeared normal (Figures 10, 11 and 12). Depending upon concentration, allicin-treated conidia showed reduced germination and abnormal hyphal morphology. These observations agree with previously reported effects of allicin on the hyphae of different fungi (thinner, collapsed hyphae with a tendency to increased vacuolization (Khan and Zhihui, 2010; Yamada and Azuma, 1977). Moreover, it was shown that the cyto-morphological modifications or changes, particularly the accumulation of lipid bodies and thickening of cell wall induced by garlic exudates are similar to those produced by some synthetic fungicides and garlic extracts (Hippe, 1991; Alberto et al., 1997; Khan and Zhihui, 2010). External wall tear and rupture in *B. sorokiniana* conidia was frequently shown at high incidence in our experiments (Figure 7). These results agreed with other investigations, indicating that observed destruction of the conidial wall structure of *F. oxysporum* subjected to garlic extract (Tariq and MaGee, 1990). The oxidation of sulfhydryl groups within the cell wall of *Candida albicans* and its impact on wall structure has also been reported (Tawfik et al., 2000).

Antifungal activities of allicin can be attributed to its interaction with the thiol group of proteins and amino acids and that, especially with the latter, allicin forms S-allyl derivatives (Gruhlke et al., 2010; Pârvu et al., 2011). Another antifungal mechanism is the allicin-mediated lipid hydroperoxide production in the fungal plasma membrane, resulting in increased permeability (Horev-Azaria et al., 2009). Allicin (diallyl thiosulfinate), the main thiosulphinate from garlic, is a volatile phytoanticipin that has been shown to be responsible for the antimicrobial effects of garlic. The inhibitory action of garlic extract on

Table 4. The effect of pure synthetic allicin on germination of conidia of *B. sorokiniana*, *S. tritici* and *D. tritici-repentis.*

Concentration of allicin (µg/ml)	Spore germination (number)	Spore germination (% of the control)	Inhibition (%)
Bipolaris sorokiniana			
80	0	0	100
60	2/100	2	98
40	37/100	41	59
20	41/100	45	55
10	67/100	74	26
0	90/100	100	0
Septoria tritici			
120	0	0	100
100	5/100	6	94
80	19/100	22	78
60	27/100	32	68
40	47/100	55	45
20	70/100	82	18
10	81/100	95	5
0	85/100	100	0
Drechslera tritici-repentis			
100	0	0	100
80	3/100	3	97
60	29/100	31	69
40	47/100	51	49
20	54/100	59	41
10	66/100	72	28
0	92/100	100	0

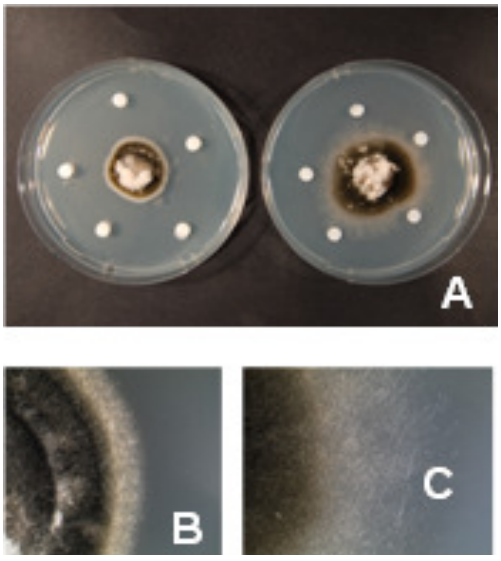


Figure 1. (A) Effect of garlic juice on radial growth of *B. sorokiniana*: left: 20 µl of garlic juice containing 53 µg allicin was pipetted onto each filter disc; right: control. Growth is shown after after 4 days incubation. (B) and (C); detail of colony edges. (B), allicin-treated; (C), control.

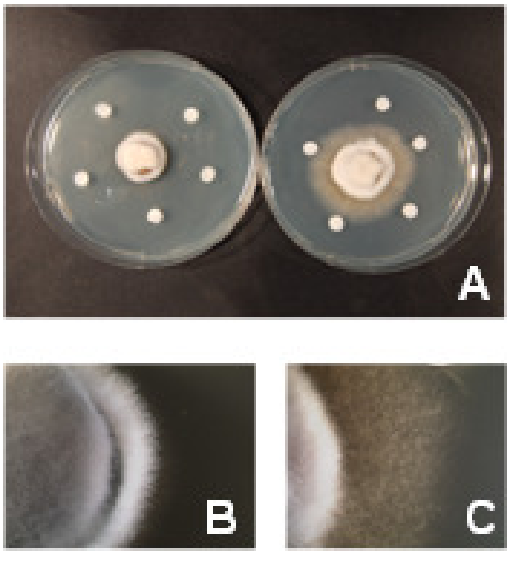
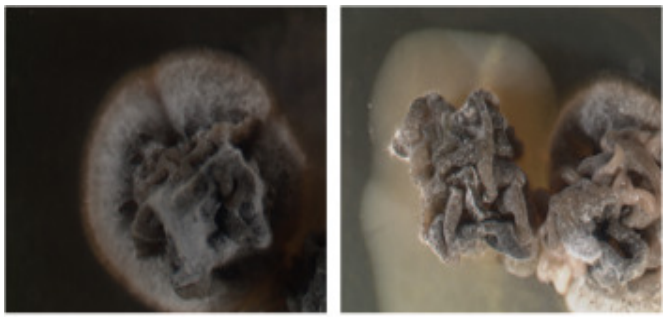


Figure 2. (A) Effect of garlic juice on radial growth of *D. tritici-repentis*: left: 20 µl of garlic juice containing 53 µg allicin was pipetted onto each filter disc; right: control. Growth is shown after after 4 days incubation. (B) and (C), detail of colony edges. (B) treated; (C), control.



(A) (B)

Figure 3. Effect of garlic juice on radial growth of *Septoria tritici*. (A) Treated with 20 µl undiluted garlic juice (53 µg allicin) pipetted onto each filter paper disc. Growth is shown after after 8 days incubation showing a stromatic type of colony growth, (B) control showing a yeast-like pink colour of secondary, actively growing conidia.

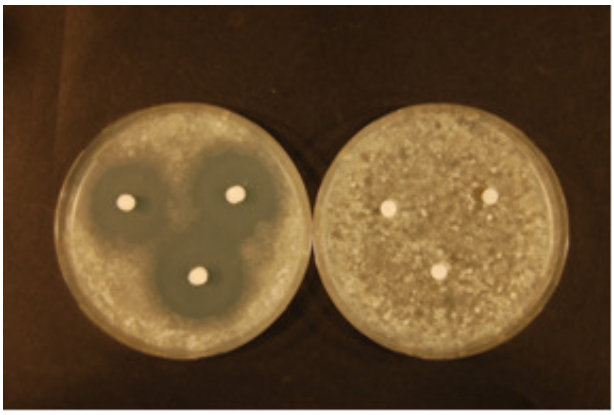


Figure 4. Inhibition of *B. sorokiniana* spore germination in seeded agar by garlic juice. Left: treatment with undiluted garlic juice (53 µg allicin per disc). Growth is shown after 4 days incubation. Right: control (20 µl of sterile distilled water per disc).

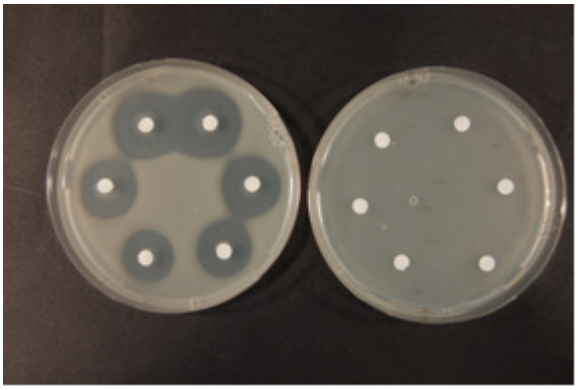


Figure 5. Inhibition of *S. tritici* spore germination in seeded agar by garlic juice. Left: treatment with 20 µl undiluted garlic juice (53 µg allicin) pipetted onto each filter paper disc. Growth is shown after 4 days incubation. Right: control (20 µl of sterile distilled water).

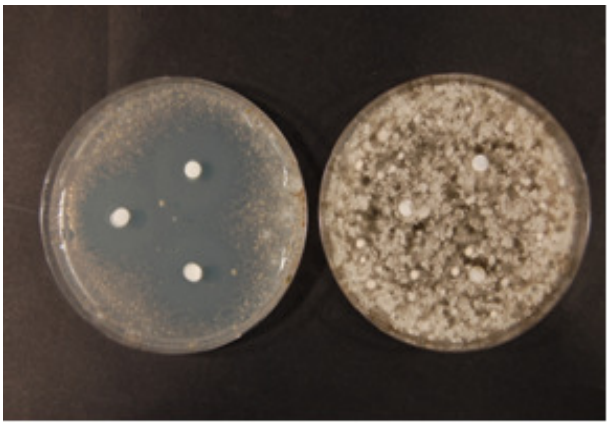


Figure 6. Inhibition of *D. tritici-repentis* spore germination in seeded agar by garlic juice. Left: treatment with 20 µl undiluted garlic juice (53 µg allicin) pipetted onto each filter paper disc. Growth is shown after 4 days incubation. Right: control (20 µl of sterile distilled water).

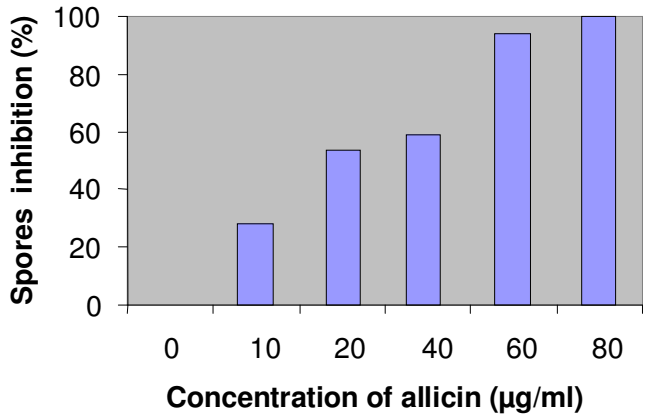


Figure 7. Effect of pure synthetic allicin on the inhibition of conidia germination (%) of *Bipolaris sorokiniana*.

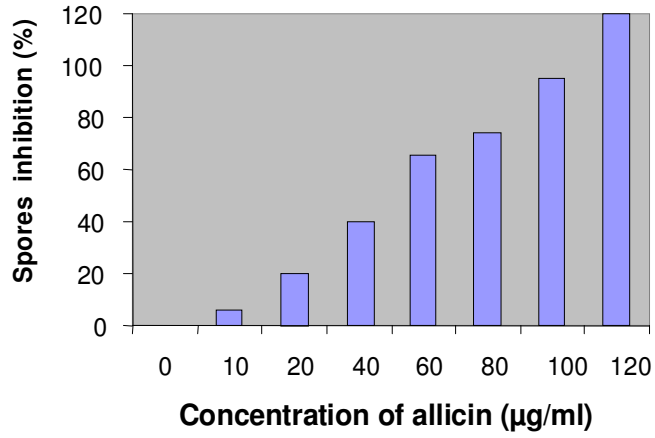


Figure 8. Effect of pure synthetic allicin on the inhibition of conidia germination (%) of *Septoria tritici*.

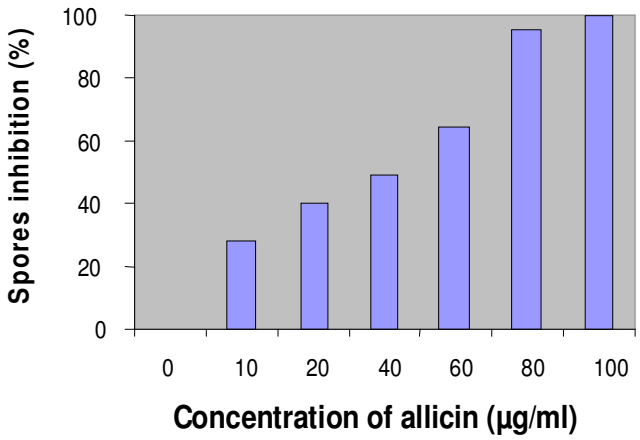


Figure 9. Effect of pure synthetic allicin on the inhibition of conidia germination (%) of *D. tritici-repentis.*

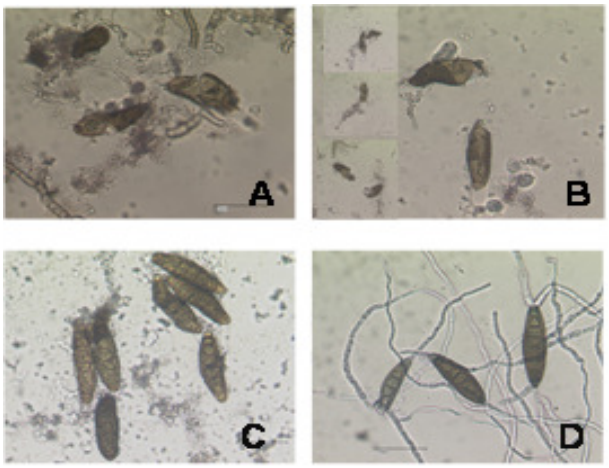


Figure 10. Abnormalities of conidia of *B. sorokiniana* induced by garlic juice treatment. (A to C) Collapsed, ungerminated conidia afterexposure to 80 µg/ml allicin; D, normally germinating conidia in the control.

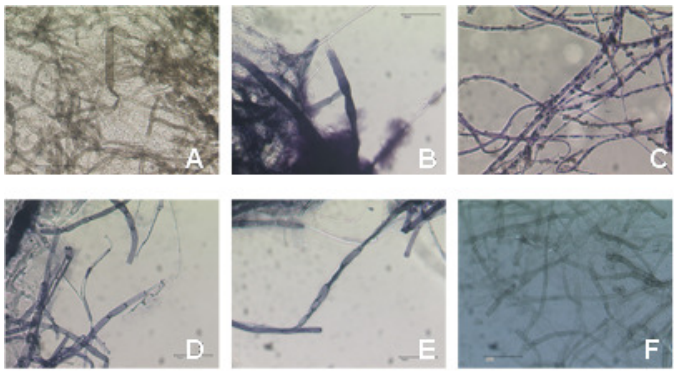


Figure 11. Abnormalities in conidia and mycelia of *D. tritici-repentis*. (A and B) Conidia and (C, D and E) mycelia of the fungus showing altered morphology and collapse leading to death after the treatment with allicin (100 µg/ml); (F) hyphae of control treatment.

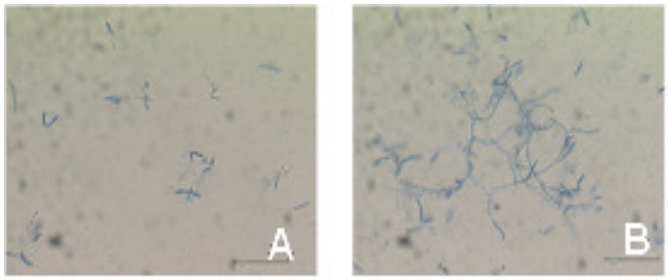


Figure 12. Conidial germination of *S. tritici*. (A) After 72 h in the presence of garlic juice (120 µg/ml allicin); (B), control.

fungal growth has been attributed to the presence of allicin as the major antifungal component (Cavallito and Balley, 1944; Muhsin et al., 2000). Furthermore, it has been reported that allicin converts into disulfhide compounds when garlic bulbs are damaged, and the volatile compounds act as fungistatic or fungicidal components that disrupt fungal cell metabolism due to the oxidation of proteins (Baron and Tansey, 1977).

Most of the medicinal effects of garlic are due to those sulfur compounds (thiosulfinates and sulfides, products of conversion of alliin from garlic by the enzyme alliinase. Analysis of allicin has been hindered because of its instability. The quality and quantity of the biologically active compounds from *Allium* sp. significantly depend on the species (Fritsch and Keusgen, 2006; Vlase et al., 2010), plant organ (Stajner et al., 2008) and the harvest time (Schmitt et al., 2005). That is why the biologically active compounds have to be determined from each plant extract. However, the different methods used in some previous reports to measure the allicin concentration from preparations of garlic extracts make comparisons difficult, regarding allicin content/effectiveness. The most promising method for analysis of allicin and degradation products (polysulfides) is reversed-phase HPLC (Blania and Spangenberg, 1991; Lawson et al., 1991a, b; Krest and Keusten, 2002).

In our study the analysis of allicin by HPLC determined 2640 to 3240 µg allicin/ml in various batches of *A. sativum* extract.

It is know that various natural products can reduce populations of foliar pathogens and control diseases development, and then these plant extracts have potential as environmentally safe alternatives and as components in integrated pest management programs (Goussous et al., 2010). A number of plant species have been reported to possess natural substances that are toxic to several plant pathogenic fungi (Arya and Perelló, 2010). Our results are in accordance with previous findings that have demonstrated the antifungal activities of garlic preparations *in vitro* and *in planta* (Yamada and Azuma 1977; Islam et al., 2001; Masum et al., 2009; Okigbo et al., 2009; Ruhul et al., 2009; Rukhsana et al.,

2010).

In the present study, we have shown that garlic extract used in this experiment has profound inhibitory effect on the growth of *B. sorokiniana*, *D. tritici-repentis* and *S. tritici*. The efficiency of allicin from garlic in controlling the fungus *Bipolaris* and *Drechslera* in other cereals like barley and rice has been examined earlier (Alice and Rao, 1987; Silva et al., 2001; Rodrigues et al., 2002; Rodrigues and Bach, 2003). In this work, we have demonstrated the antifungal activity of garlic juice against the necrotrophic complex of wheat pathogens.

Conclusions

The results from our study suggested that garlic extract affected germination of spores of the three fungal wheat pathogens tested with noticeable inhibition of the growth, spore germination, and induced modifications in the morphology or structure of hyphae and conidia. Restriction of the radial growth of the fungal colonies suggests a good antifungal effect even up to 10 days. The level of garlic juice necessary to inhibit spore germination was not the same as that needed to inhibit hyphal growth. Moreover, regarding the broad spectrum of the pathogens directly inhibited by allicin from garlic extract, and the known effect of allicin and garlic juice in inducing plant defence responses (Silva et al., 2001), results support the use of garlic extract as a useful, cost-effective and environmentally friendly management strategy in controlling the leaf-spotting complex in wheat plants, with the purpose to minimize the use of fungicides. Its advantages are its simplicity and safety.

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