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EFFECT OF GARLIC EXTRACT ON SEED GERMINATION, SEEDLING HEALTH, AND VIGOUR OF PATHOGEN-INFESTED WHEAT

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Abstract: The effect of garlic extract containing bioactive allicin on the germination and subsequent seedling vigour of pathogen-infested wheat seeds, was tested. The first aim was to characterize the antifungal activities of garlic extract and pure allicin, on the most frequently occurring wheat pathogens of the *Helminthosporium* genus (*sensu lato*) in Argentina. The second aim was to characterize the antifungal activities of garlic extract and pure allicin on moulds belonging to the natural endogenous microflora. Garlic extract showed fungicidal activity on the endogenous fungal contamination of the wheat seeds and particularly reduced the degree of disease caused by *Bipolaris sorokiniana* and *Drechslera tritici-repentis*. Allicin in garlic juice corrected the poor germination of wheat seeds caused by natural mycoflora of grain. Growth promoting activities of garlic juice on wheat seedling vigour was reported. Interestingly, the inoculum on naturally infected wheat seeds could be reduced with garlic juice as a seed dressing biofungicide, before sowing. In this study, we demonstrated the efficacy and the high control potential of garlic extract against seed-borne wheat fungi. Such results suggest that using garlic extract can minimise the risk of infection as well as minimise the risk of chemical fungicide exposure. On the basis of these results, scale-up to field trials using garlic extract and allicin as the dressing biofungicide before sowing for disinfection of wheat seeds, seems justified as a sustainable alternative to the use of chemical fungicides.

Key words: antifungal activity, biofungicides, fungal diseases, garlic, sustainable agriculture, wheat

INTRODUCTION

Important or devastating crop diseases are often seed-borne and caused by fungi. In addition, it has been demonstrated that seed-borne fungi are responsible for the poor quality of seeds in many crops (Neergaard 1977). On wheat, seed-borne pathogens causing plant diseases include *Bipolaris sorokiniana* (Bs) (Sacc.) Shoemaker (teleomorph *Cochliobolus sativus* Ito & Kurib., Drechsler ex Dastur), and *Drechslera tritici-repentis* (Dtr) (Died.) Shoemaker (teleomorph *Pyrenophora tritici-repentis* (Died.) Drechsler). The above are considered the most frequently occurring fungi of the *Helminthosporium* genus (*sensu lato*). These fungi infect all parts of the plant, causing spot blotch and tan spot, respectively (Dubin and Duveiller 2000). Spot blotch dominates in the warmer, humid areas of Argentina. Tan spot prevails in cooler seasons in areas where wheat is grown organically (Duveiller *et al*. 1998). These pathogens are carried on seeds or within seeds, and can reduce seed germination and seedling emergence (Özer 2005). Seed-borne pathogens may cause seed abortion, seed rot, seed necrosis, reduction or elimination of germination capacity as well as seedling damage. Disease at later stages of plant growth by systemic or local infection can take place (Bateman and Kwasna 1999; Khanzada *et al*. 2002).

Many seed-borne fungi of wheat are generally managed by synthetic chemicals, which are considered both efficient and effective. But, the continuous use of these fungicides started unraveling non-biodegradability and is known to have residual toxicity causing pollution (Torp *et al*. 2006). Moreover, seed-borne diseases can cause serious problems in organic cereal production because no chemical treatments can be used, and so far, only a few alternative treatments have been approved (FAO 2004). Among the recent alternative strategies used for plant disease management, there has been great success achieved using plant-derived products (Gulter 1988; Arya and Perelló 2010). The antifungal activity of many plant-derived products against a wide range of phytopathogens has frequently been documented. Moreover, the efficacy of the antifungal properties of some herbaceous and medicinal plants against cereal seed-borne mycoflora, was recorded (Alice and Rao 1986; Silva *et al*. 2001; Rodrigues 2002; Rodrigues *et al*. 2003; Antoniazzi *et al*. 2008; Masum *et al*. 2009; Kiran *et al*. 2010; Yassin *et al*. 2012). Among many plant substances generally used against seed-borne mycoflora, garlic is very promising considering its highly significant antifungal activity and antimicrobial properties (Harris *et al*. 2001; Obagwu and Korsten 2003; Benkeblia 2004; Onyeagba *et al*. 2004; Grozaw and Foarce 2005;

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Haciseferogullari *et al.* 2005; Irkin and Korukluoglu 2007; Aqil *et al*. 2010; Reddy *et al*. 2010). Studies of garlic's activity on wheat fungi are limited (Khansada *et al*. 2002; Hasan *et al*. 2005). Garlic, though, has been frequently documented on other fungal pathosystems (Islam *et al*. 2001; Ismaiel 2008; Okigbo *et al.* 2009; Ruhul *et al.* 2009; Rashid *et al.* 2010; Salim 2011; Tagoe *et al*. 2011).

The present work aimed to assess the effect of seed applications of garlic juice (GJ) against: (1) the naturally seed-borne mycoflora of wheat, (2) two target fungi causing wheat grain disease, *B. sorokiniana* and *D. tritici-repentis,* and (3) the present work to evaluate the range of growth-promoting activities of garlic juice on seedling emergence and vigour of wheat.

A report about the *in vitro* efficacy of garlic to control fungal pathogens of wheat has previously been published (Perelló *et al*. 2013).

MATERIAL AND METHODS

Collection of seed samples

Wheat seeds samples of cultivars Klein Zorro, Buck Guapo, and Klein Escorpión commonly grown in the Buenos Aires Province of Argentina, were collected from the commercial lots that were used for sowing in the farms. Then, the samples were used in the experiments. Until being used, seeds were stored at room temperature (aprox 20°C, dry storage) at the Department of Plant Physiology (BioIII), RWTH Aachen University, Germany, where the present investigation was conducted.

Garlic juice preparation

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. A domestic juicer (Turmix Fabr. No. 1068; Turmix AG, Jona, Switzerland) was used to extract the juice. The juice was poured into a sterile 50-ml Falcon tube and centrifuged at 5000 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; Vacubrand 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 was determined by HPLC and it was used immediately after being appropriately diluted (264 μg/ml, 132 μg and 66 μg/ml). Dilutions were carried out using deionized water.

Quantitative analysis of allicin in garlic juice, by HPLC

The method used was based on that of Krest and Keusgen (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 were injected into the HPLC (JASCO system with UV-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. An RP-C18 column (Machery and Nagel, Dueren, Germany) was used for separation and the chromatogram detection was at 254 nm.

Seed infection evaluation

Seeds were divided into two equal parts. The first part was made up of non-surface sterilized seeds. The second part was made up of seeds disinfected by soaking in 1% sodium hypochlorite for 1 min. The seeds were used in two experiments, as shown below: agar test and paper towel (rolled towel) method.

Initial seed health testing was conducted by the agar plate method (Anonymous 1976). Ten seeds were placed in each Petri plate containing 20 ml of Potato Dextrose Agar medium (PDA). Incubation was done at 20±2°C for seven days. Four replications were made. A total of 80 seeds were analyzed in both experiments.

After incubation, the Petri dishes were examined for fungal growth under a stereo-binocular microscope. In further experiments, seeds were evaluated for the presence of *B. sorokiniana* and *D. tritici-repentis.* Isolation of both pathogens was performed for non-disinfected or disinfected seeds as mentioned above.

Identification of the used fungi isolates

Bipolaris sorokiniana (Bs) and *D. tritici-repentis* (Dtr) were isolated from seeds and purified using the single spore isolation technique and the hyphal tip method. Identification was done based on the conidial morphology examination and colony characters referring to the Illustrated Genera of Imperfect Fungi (Barnett and Hunter 1972) and Demataceous Hyphomycetes (Ellis 1971). Isolates of both fungi were maintained at 5°C on PDA and V8 agar, for further studies.

Biological assay

The biological activity of GJ on naturally infected wheat seeds (native mycoflora) and on the inoculated wheat seeds with the two target pathogens, *B. sorokiniana* and *D. tritici-repentis*, was assessed in the laboratory on the wheat cultivars K. Zorro, B. Guapo, and K. Escorpión.

Treatments were: (a) seeds treated with fungicide (Thiram®); (b) seeds sprayed with water; (c) pathogen-inoculated seeds (Bs and Dtr, separately); (d) GJ treated seeds at concentrations of 1:5, 1:10, and 1:20 (264 μg/ml, 132 μg and 66 μg/ml); (e) seeds treated with GJ and inoculated with conidial suspensions of Bs (2×10^4 conidia/ml) after 24 h; (f) seeds treated with GJ and inoculated with conidial suspensions of Dtr (3×10^4 conidia/ml) after 24 h. Seeds in groups (e) and (f) were sprayed with garlic juice preparations. After 24 h, at room temperature, with a 12 h photoperiod, seeds in groups (e) and (f) were inoculated with conidial suspensions, by spraying.

Agar test

For the agar test, four replicates were used. Each replicate consisted of ten seeds/Petri dish (9 cm diam.) containing PDA 2% of treated seeds, and the untreated control/treatment from each of the three wheat cultivars, set up in a growth chamber at 20±2°C and 12 h light-darkness for 7 days. The following parameters were assessed: count of seedling emergence, natural seed-borne infection, *B. sorokiniana* infection and *D. tritici-repentis* infection. All of these parameters were recorded 7 days after sowing and expressed as a percentage. The seedling percent emergence was calculated as done by Baset Mia and Shamsuddin (2009). Emergence of a radicle of 5 mm was used as the criterion for germination.

Paper towel (Rolled towel) method

The paper towel method (Neuman *et al.* 2010) was used to know the effect of seed-borne inoculums on seed quality parameters of wheat *i.e.* to carry out germination and vigour tests. Forty randomly selected seeds were placed on four long lanes on moistened paper sheets; ten seeds were arranged per lane, in four lanes. The seeds were rolled carefully to avoid any excess pressure on seeds. This experiment was carried out under growth chamber conditions, at temperatures ranging between 25–29°C, and 12 h light – 12 h darkness. All the seedlings were counted and the percentage of germination was calculated. To find out the seedling vigour, normal seedlings were taken from the germination test at random. The root length (cm) was measured. The same seedlings were used for the measurement of the shoot length (cm). In addition, the root numbers were recorded. Vigour index was calculated 21 days after sowing according to the formula of Randahawa *et al.* (1985):

$$VI = SG \times (SL + RL)$$

where:
VI – vigour index,
SG – seed germination (%),
SL – mean shoot length (cm),
RL – mean radicle length (cm),
(SL + RL) – seedling length.

Statistics

Data were analysed using a Kruskal-Wallis one-way AnovaR on ranks, combined with Dunn's method for pairwise comparison.

RESULTS AND DISCUSSION

Percent seedling germination for all three wheat cultivars tested was > 85% and was greatly increased by either fungicide or garlic juice treatments depending on the wheat cultivar tested (Fig. 1A, B, C).

Treatment with Thiram® and garlic juice significantly reduced the degree of contamination depending on cultivar and garlic juice containing allicin (Fig. 2A, B, C). The highest concentration of garlic used (1000 μg/ml) reduced the degree of contamination to more than 50% in the cultivars K. Zorro and B. Guapo. At the tested concentrations of 500 μg/ml allicin, there was a significant reduction in the seed-borne mycoflora of the cultivar Klein Escorpión (Fig. 2C).

It was observed that the three varieties tested, differed significantly from one another. This variation might be due to: (1) the effect of *B. sorokiniana* on grains, (2) the effect of

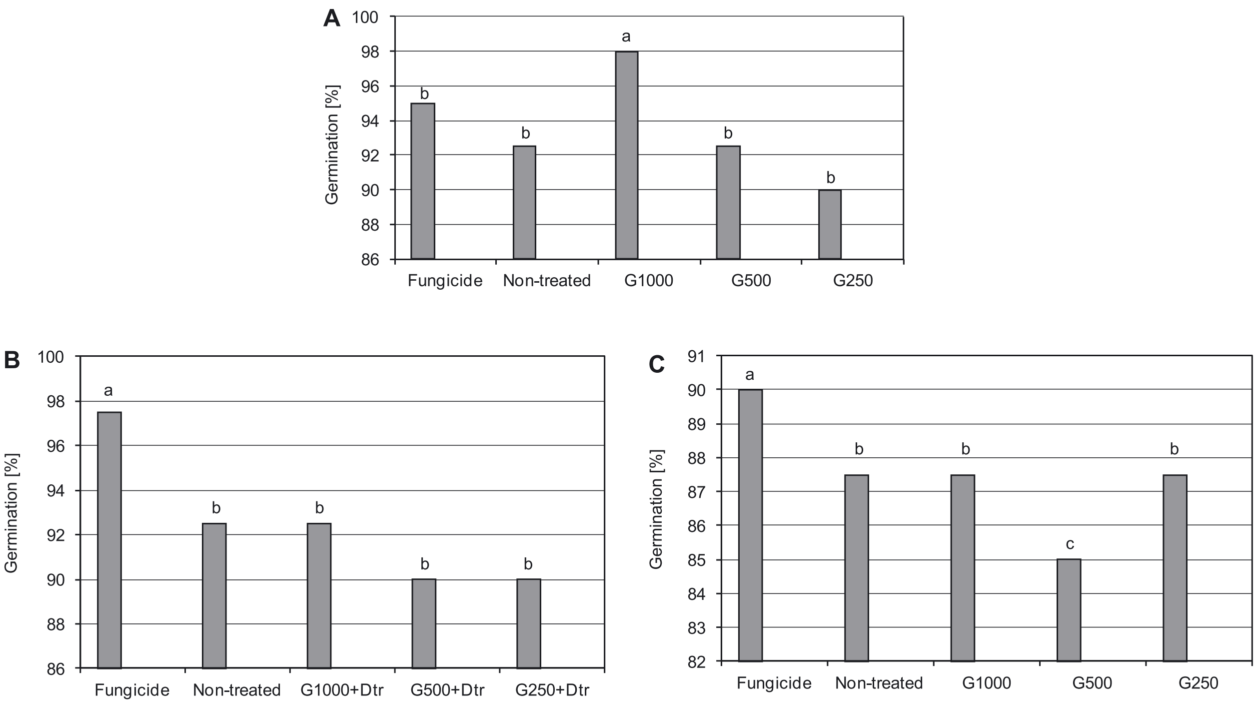

Fig. 1. Seed germination, in wheat cultivars treated with different concentrations of allicin in garlic juice. A – cultivar Klein Zorro; B – cultivar Buck Guapo; C – cultivar Klein Escorpión. Significant differences ($p < 0.05$) between means were indicated by different letters above histogram bars

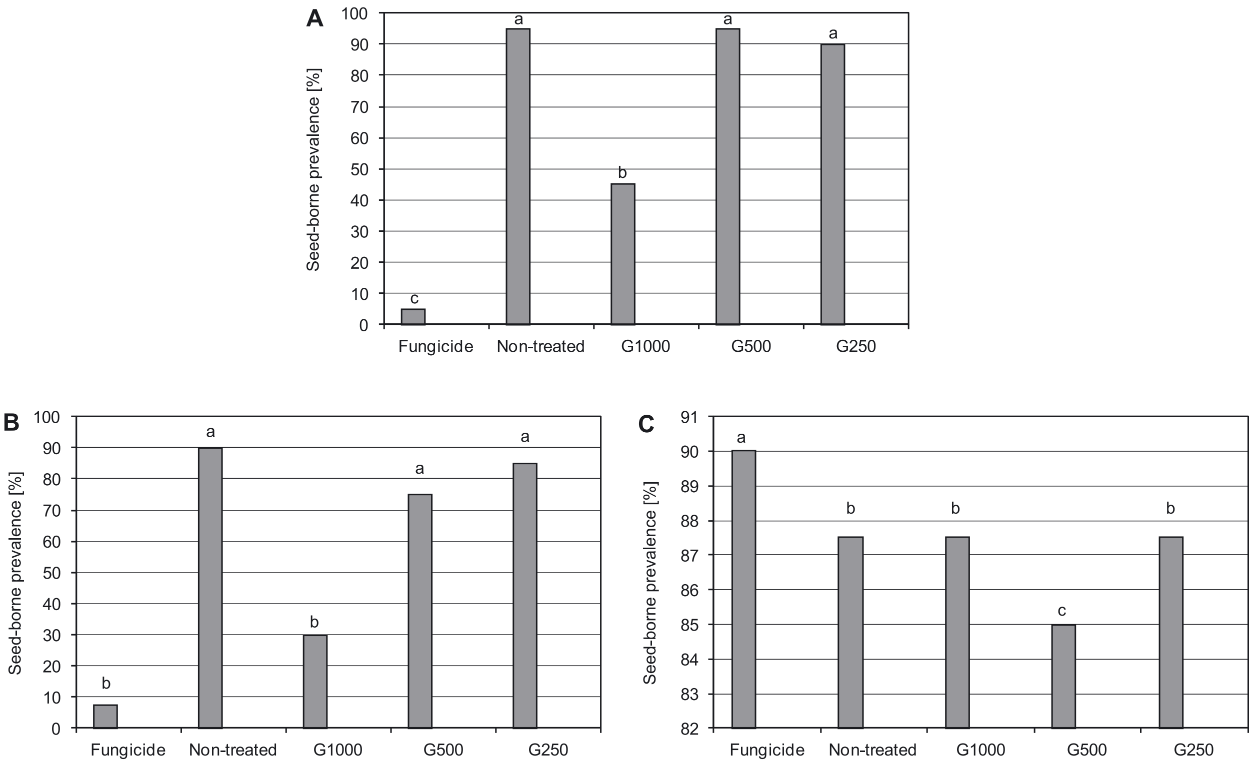

Fig. 2. Seed-borne prevalence in wheat cultivars treated with different concentrations of allicin in garlic juice. A – cultivar Klein Zorro; B – cultivar Buck Guapo; C – cultivar Klein Escorpión. Significant differences ($p < 0.05$) between means were indicated by different letters above histogram bars

D. tritici-repentis on wheat, (3) the effect of the natural seed-borne native mycobiota on wheat seeds, (4) variation of the genetic make-up of wheat material, among other reasons.

Neither Thiram® nor the application of garlic juice had a significant influence on the number of roots produced by the tested cultivars. The root lengths for three tested cultivars were not different between the controls and Thiram® treatments. Qualitatively, however, the non-treated seedlings had the highest occurrence of deformed and diseased roots compared to the treatments with Thiram® or garlic juice. Wheat seeds without the fungicide or garlic juice treatment showed a higher prevalence of pathogenic symptoms, such as root rot, shorter roots, blackened roots, and softening and necrosis of tissues. This confirms the association of seed-borne fungi with seed viability, wilting of plants, and stem flaccidity.

Garlic juice treatment led to significantly greater shoot length depending on the wheat cultivar and garlic juice concentration tested, compared to K. Escorpión and the untreated controls (Fig. 3A, B, C). The vigour of seedlings was significantly higher in the cv. Buck Guapo at three tested concentrations, compared to the untreated controls. The vigour of the seedlings of cvs K. Zorro and K. Escorpión was not affected or was even slightly reduced with GJ treatments (Fig. 4A, B, C).

Effect of seed treatment in controlling *B. sorokiniana*

Inoculation of Klein Escorpión seeds with *B. sorokiniana* significantly reduced the germination rate in comparison with the rest of the treatments (Table 1). Interestingly, both fungicide and GJ applications significantly improved the seed germination although the great inoculum pressue of the pathogen. The seed-borne infection was also significantly corrected by Thiram® and by garlic juice treatments. Over a 40–50% reduction in infection was obtained by the application of these treatments at three concentrations. An infection reduction of more than 50%, and better control of the pathogen, was achieved with the Bs+GJ1000 application. Shoot length was positively influenced by Thiram® or garlic juice treatments. The positive effect of garlic improving the germination rate and shoot length was significantly reflected in the overall vigour index of seedlings.

Effect of seed treatment in controlling *D. tritici-repentis*

Inoculation of Klein Escorpión seeds with *D. tritici-repentis* scarcely modified the germination rate. Although fungal inoculation reached more than 80% of seed infection (80, 48%), this effect was positively corrected by Thiram® and by garlic juice applications that significantly reduced the Dtr prevalence. Shoot and root length were not influenced by either fungicide or garlic juice treatments. In fact, the application of the Dtr+GJ1000 treatment slightly reduced both the root and shoot length of seedlings. Differences in seedling vigour were not detected in the control (Dtr) and Dtr+GJ500/Dtr+GJ250 treatments, or vigour was significantly lower (Dtr+GJ 1000) than the control (Dtr) (Table 2). This could indicate a more sensitive behaviour of this pathosystem against a high alicin in GJ concentration.

On the other hand, the positive effect of the fungicide improving vigour and germination (%) as well as significantly reducing the seed-borne prevalence, was significantly demonstrated.

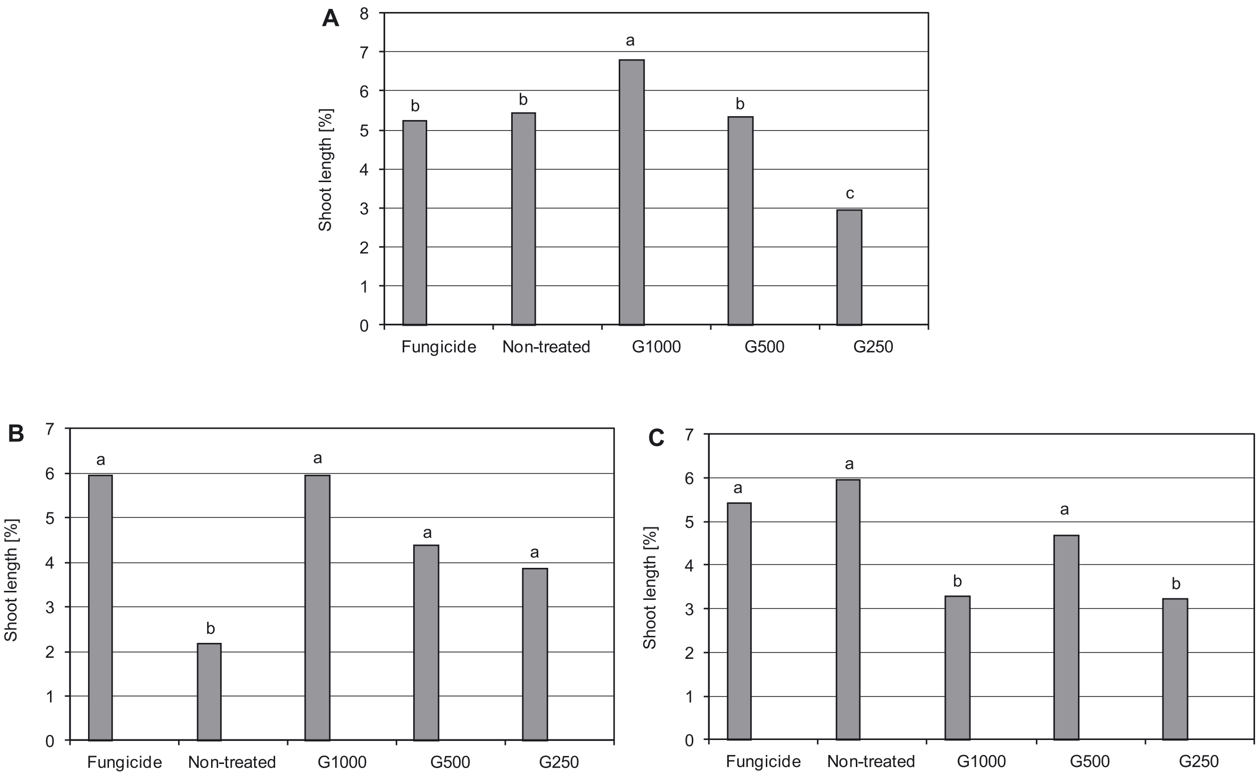

Fig. 3. Shoot length in wheat cultivars treated with different concentrations of allicin in garlic juice. A – cultivar Klein Zorro; B – cultivar Buck Guapo; C – cultivar Klein Escorpión. Significant differences ($p < 0.05$) between means were indicated by different letters above histogram bars

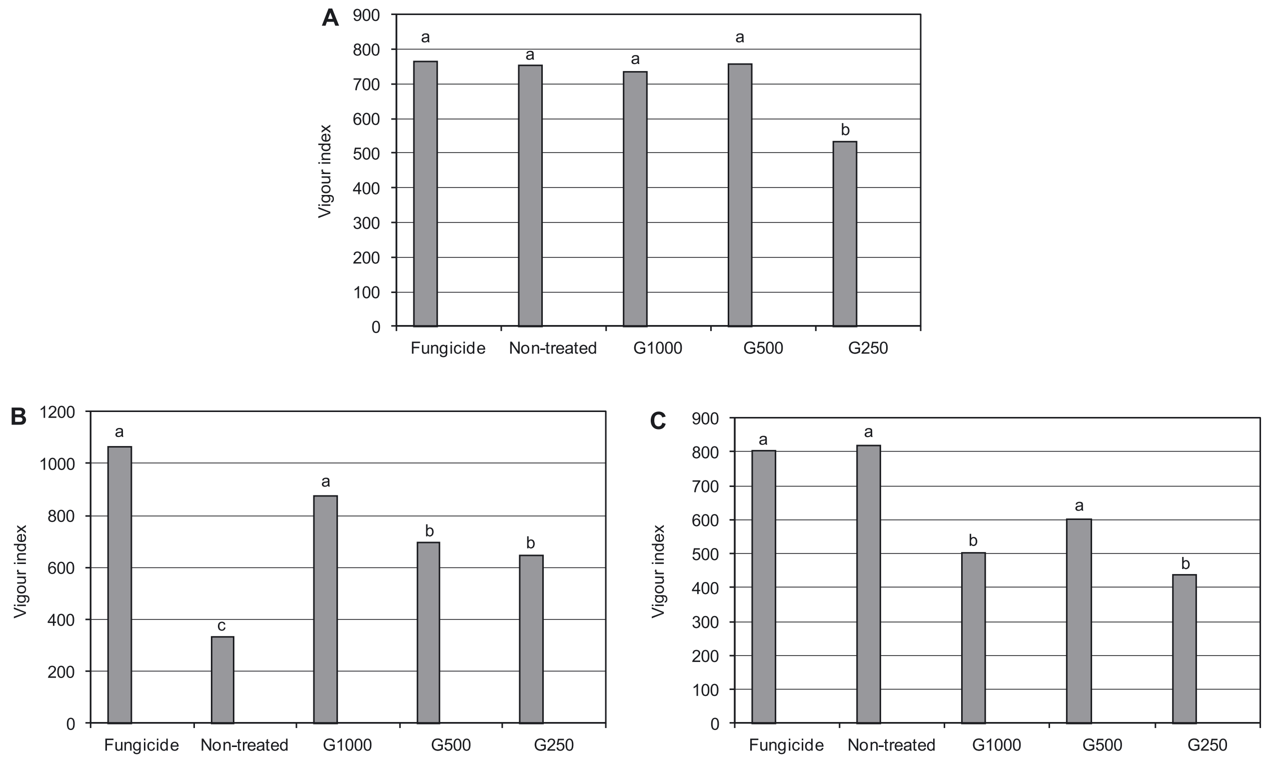

Fig. 4. Vigour of seedlings in wheat cultivars treated with different concentrations of allicin in garlic juice. A – cultivar Klein Zorro; B – cultivar Buck Guapo; C – cultivar Klein Escorpión. Significant differences ($p < 0.05$) between means were indicated by different letters above histogram bars

Table 1. Effect of seed treatment on the seed germination, infection, and seedling vigour of wheat cultivar K. Escorpión treated with different concentrations of garlic juice before fungal inoculation of *B. sorokiniana* (Bs)

Treatment	Germination [%]	Bs Prevalence [%]	No. roots	Root length [cm]	Shoot length [cm]	Vigour index
Thiram	80.00 a	0 c	5.37 a	3.50 a	5.41 a	712.80 a
Control (Bs)	60.00 c	95.0 a	4.59 a	1.52 b	1.97 c	209.40 c
Bs+GJ1000	72.50 b	42.8 b	4.83 a	3.43 a	3.68 b	515.47 b
Bs+GJ500	72.50 b	55.0 b	4.89 a	3.49 a	3.79 b	520.55 b
Bs+GJ250	67.50 b	50.0 b	5.20 a	1.82 b	2.66 b	302.40 c

Different letters indicated significant differences ($p < 0.05$)

Table 2. Effect of seed treatment on the seed germination, infection, and seedling vigour of wheat cultivar K. Escorpión treated with different concentrations of garlic juice before fungal inoculation of *D. tritici-repentis* (Dtr)

Treatment	Germination [%]	Dtr Prevalence [%]	No. roots	Root length [cm]	Shoot length [cm]	Vigour index
Thiram	90.00 a	0 c	5.37 a	3.51 a	5.41 a	801.90 a
Control (Dtr)	65.00 b	80.48 a	4.33 a	2.27 b	5.77 a	522.60 b
Dtr+GJ1000	57.50 c	34.00 b	5.13 a	1.15 c	1.38 b	145.47 c
Dtr+GJ500	72.50 b	44.18 b	4.75 a	2.09 b	5.24 a	531.42 b
Dtr+GJ250	65.00 b	69.56 b	5.10 a	2.58 b	5.68 a	536.90 b

Different letters indicated significant differences ($p < 0.05$)

CONCLUSION

This study confirmed that natural mycoflora present in wheat grain was capable of causing poor seed germination and was capable of negatively influencing seedling growth. Our study also confirmed that this effect could, depending on the cultivar, be corrected by treatment with fungicide (Thiram®) or treatment with garlic juice containing allicin. Interestingly, the seedling vigour index was not always improved after fungicide or garlic juice treatments. Such results suggest that although the infection load was reduced, this did not always lead to better seedling performance. However, this may depend to some extent on the cultivar, since such an improvement was clearly demonstrated for Buck Guapo.

It was also confirmed, that the two target pathogens *B. sorokiniana* and *D. tritici-repentis* inoculated at high inoculum density caused the greatest reduction in germination percentage and seedling vigour. Seeds treated with garlic juice had a relatively better germination percentage, plumule and radicle length, and seedlings, than those seeds treated with high inoculum density of the target pathogen *B. sorokiniana*. Thus, the inoculum level on naturally infected wheat seeds could be reduced through the use of garlic juice as the seed dressing biofungicide, before sowing. This could greatly improve radicle and plumule development of wheat seeds, particularly when *B. sorokiniana* is involved in the seed infection. From the findings of the present study, it has been suggest that investigation of garlic products to develop fungicidal seed treatments should be further investigated.

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