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ARTICLE

Effectiveness of the antifungal black seed oil against powdery mildews of cucumber (*Podosphaera xanthii*) and barley (*Blumeria graminis* f.sp. *hordei*)

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ABSTRACT When cucumber and barley leaves were sprayed with 0.5% black seed oil (BSO), rapeseed oil (R oil) and parafine oil (P oil), disease severity of the powdery mildew of cucumber (*Podosphaera xanthii*) was reduced from 52% (control) to 7.7% (BSO), 18.6% (R oil) and 20% (P oil). Similarly the disease severity of barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) was greatly reduced from 63.4% (control) to 9.4% (BSO), 16% (R oil) and 16.4% (P oil). Oils inhibited the conidial germination of cucumber and barley powdery mildews to 29-30.7, 35-38 and 37-41% respectively, as compared to control (58-65%). Furthermore, mycelial growth of the pathogen was severely restricted after application of BSO and other oils. Levels of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) as well as the activity of the antioxidant enzymes in the treated leaves with oils and untreated (control) were measured and determined. H_2O_2 and $O_2^{\cdot-}$ levels slightly increased, however some antioxidants are decreased such as dehydroascorbate reductase (DHAR) but other enzymes were increased such as ascorbate peroxidase (APX) and glutathione S transferase (GST). It can be concluded that the protective effect of oils against powdery mildews resulted mainly from the inhibition of conidial germination and suppression of the mycelial growth of the pathogens and there is slight activation of the host defence mechanisms. Therefore, it is important to giving more attention to BSO and other oils which have effectiveness against powdery mildew pathogens as an alternative control methods which safety and suitable for healthy and organic food production.

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KEY WORDS

black seed oil
powdery mildew
barley
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Powdery mildew disease is one of the most serious plant diseases, causing large yield losses in a number of crops (Kiss 2003). Powdery mildew, caused by *Podosphaera xanthii* [syn. *P. fusca* (Castagne) U. Braun & Shiskoff, *Sphaerotheca fuliginea* (Schlecht.) Pollacci], is a serious disease affecting the leaves, stems and fruits of cucumber (*Cucumis sativus* L.) grown in greenhouses and in the field (Bettiol et al. 2008). Powdery mildew of cucumber is one of the most dangerous foliar diseases, attacking cucumber plants in Egypt and other countries (Harfoush and Salama 1992; Mosa 1997; Reuveni et al. 1997; Verhaar et al. 1997).

Powdery mildew fungi (Ascomycotina) infect and cause substantial economic losses on a wide range of mono and dicotyledonous species (Bushnell 2002). They are obligate biotrophs, growing and reproducing entirely on living epidermal cells and obtaining nutrients from their host by means of intracellular feeding structures known as haustoria (Yarwood 1957).

The disease is controlled in commercial cucurbits by means of frequent applications of fungicides (Kimati et al. 1997). The use of fungicides has resulted in the development of *P. xanthii* populations in which resistant to fungicides (McGrath 1996; McGrath et al. 1996), and have raised public concerns over contamination of the environment and foods. The identification of biocompatible products for managing cucurbits powdery mildew with low animal toxicity and low potential risk to the environment would be a valuable contribution to disease management (McGrath and Shiskoff 1999). Disease severity has been effectively reduced by mineral oils (Daughtrey et al. 1993), plant oils (Locke and Stave 1992) and other materials applied to cucurbits (Ziv and Zitter 1992; Steinhauer and Besser 1997).

Blumeria graminis f.sp. *hordei* (*Bgh*) causes one of the most significant diseases of barley if it is not controlled by fungicides or resistant varieties. Due to its economical importance and its exquisite developmental biology (Zhang et al. 2005), *Bgh* is the most intensively studied of all powdery mildew fungi (Both et al. 2005) and is a model organism for research on obligate biotrophic fungal pathogens (Skamnioti et al. 2008).

The use of alternative control methods against *Bgh* by spraying hydrogen peroxide (H_2O_2) low and moderate con-

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Figure 1. Effect of black seed oil (BSO) on the symptoms of cucumber (right) and barley (left). Leaves inoculated with powdery mildew pathogen and sprayed with BSO three times. Control leaves sprayed with water + 5% Tween 20. Symptoms were photographed 2 days after third spray of cucumber and barley.

centrations were very effective to suppress the fungal growth and inhibiting the spore germination. Interestingly enough that H_2O_2 did not affect negatively barley leaves (Hafez and Kiraly 2003; Hafez, 2005).

A number of alternative methods have been developed to control fungal diseases, including mineral salts, essential oils, plant extracts and biological agents (Gyung et al. 2004).

Black cumin (*Nigella sativa* L.) is an annual dicotyledon of the Ranunculaceae family, grown in countries bordering the Mediterranean Sea. It has been employed for thousands of years as a food preservative and as a medicinal plant (Agarwal et al. 1979, Rahman et al. 2001). In Egypt, it is one of the widely distributed native plants (Farid et al. 2000). The seeds of black cumin are used in folk (herbal) medicine in all over the world for the treatment and prevention of a number of diseases and conditions that include asthma, diarrhoea and dyslipidaemia (Ali and Blunden 2003). The seeds contain both fixed and essential oils, proteins, alkaloids and saponin. Most of the biological activity of the seeds has been shown to be due to thymoquinone, the major component of the essential oil, which is also present in the fixed oil (Ali and Blunden 2003).

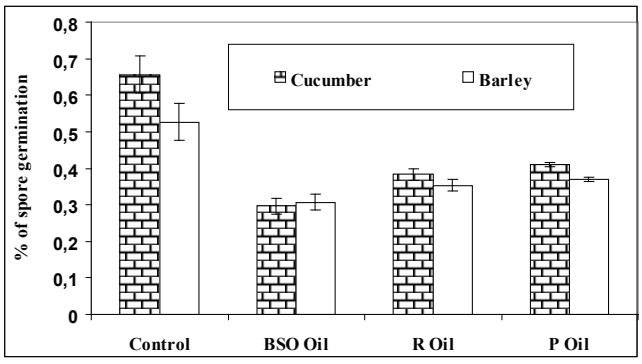


Figure 2. Rate of spore germination of cucumber powdery mildew (*Podoshaera xanthii*) and barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) on cellophane treated with oils 20 min after inoculation. Control: celphane membranes were treated with Tween 20, BSO: black seed oil, R: rapeseed oil, P: parafine oil. Results were evaluated one day after treatment.

It has been shown that the *N. sativa* oil exhibited strong antimicrobial activity against phytopathogenic fungi, *i.e. Pythium vexans, Rhizoctonia solani* and *Colletotrichum capsici* (Rathee et al. 1982). Recently, it was indicated that black seed oil possesses significant antibacterial activity against *Listeria monocytogenes* (Manoj et al. 2005).

Furthermore, the antifungal activity of black seed oil (BSO) against *Rhizoctonia solani, Botrytis fabae, Fusarium solani, Alternaria tenuis* and *Sclerotinia sclerotiorum* was investigated by Rahhal (1997). He found that the essential oil of *N. sativa* was effective against all tested fungi except *B. fabae*. Farid and others (2000) have demonstrated that BSO can control squash powdery mildew disease caused by *Erysiphe cichoracearum*.

Here I provide evidences that, BSO and other oils can suppress the powdery mildews of cucumber (*Podosphaera xanthii*) and barley (*Blumeria graminis* f.sp. *hordei*). To understand the mechanisms behind the effect against powdery mildew, toxicity test, histochemical staining were conducted *in vivo* for H_2O_2, and $O_2^{\cdot-}$ as well as biochemical assays of some antioxidant enzymes. Also, the spore germination and fungal growth were analyzed after treatment with oils using a microscopic examination.

The aim of this study is to control the powdery mildew diseases in cucumber and barley using BSO and other oils. In addition, what about the mechanism of plant oils in inhibiting the powdery mildew fungus? Is it direct toxic effect only or it is inducing resistance?

Materials and Methods

Plant hosts and pathogens

Powdery mildew-susceptible cucumber (*Cucumis sativus* L. cv. Budai csemege) and barley (*Hordeum vulgare* L. cv. Botond), seeds were sown in 12-cm plastic pots containing

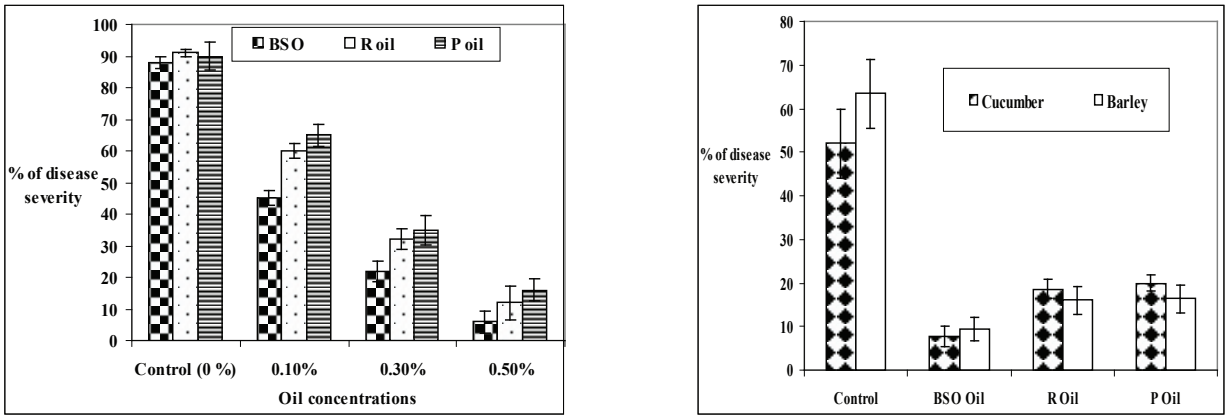


Figure 3. Disease severity of powdery mildews infected cucumber (*Podoshaera xanthii*) and barley (*Blumeria graminis* f. sp. *hordei*). Disease severity was measured 2 days after the third spray with black seed (BSO), rapeseed (R) and parafine (P) oils. Percentage (%) of disease severity of different concentrations of oils was also measured (0, 0.1, 0.30 and 0.50 %). Means of three independent experiments are shown. Error bars present ± SD.

soil mixed with peat moss (1:1) and grown in the greenhouse of the Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary. Temperature was 18-23°C, with 12-14 hours photoperiod per day using supplemental light with a light intensity of 160 μE m^{-2} s^{-1} and relative humidity 75-80%.

Cucumber powdery mildew *Podoshaera xanthii* and Barley powdery mildew *Blumeria graminis* f.sp. *hordei* race A6 were maintained in phototron chamber and used for the inoculations. Inoculum was dispersed in the greenhouse atmosphere by placing plants of cucumber and barley bearing sporulating colonies of *P. xanthii* and *Blumeria graminis* f.sp. *hordei* race A6 beneath ventilation fans of the greenhouse (Kiss et al. 2001; Hafez and Kiraly 2003; Bettiol et al. 2008).

Treatments of the plants with oils and inoculation with pathogens

Black seed oil (BSO) from *Nigella sativa* was obtained from trade market in Egypt, rapeseed oil was obtained from trade market in Hungary and parafine oil was obtained from the laboratory. For emulsification, a test tube containing 5 ml oil, 4.5 ml distilled water and 0.5 ml Tween 20% was shaken vigorously 20 times by hand. The mixture was dilluted with distilled water to make 0.1% oil emulsion. Water + 0.01% Tween 20 was used as the control. When 0.5% oil was used the concentration of Tween 20 was also increased to 0.05% (Farid et al. 2000; Ko et al. 2003). Application with oils was conducted 2rd and 3rd days after the inoculation.

The designed plants (4 to 6 weeks old) were sprayed three times to runoff with the plant oils solutions using a sprayer bottle every two days. Leaves sprayed with Tween 20 were used as the control. Treated plants placed on a bench were surrounded with cucumber and powdery mildew pathogens (Kiss et al. 2001) as the inoculum source. Five plants with fully expanded leaves were used for each treatment. All the experiments were performed three times.

Toxicity test and disease severity

To determine the inhibitory effect of oils, a cellophane water agar media (15 g agar and 1000 ml distilled water) was used. Before pouring the agar medium in Petri dishes, 5 ml/L chloramphinicol antibiotic (1% in ethanol) was added to the media. A cellophane membrane (well washed in boiling distilled water) was placed on the surface of water agar (Chet et al. 1981). The spores were distributed with sterilized brushes on the surface of the cellophane and then sprayed gently with oil solutions. Control Petri dishes contained only spores treated with Tween 20. After 18-24 hours, the rate of spore germination was tested by using Olympus BX 51 (Germany) microscope.

Estimation of infected leaf area on each leaf covered with powdery mildew was made 2 days after the third spray. The severity of powdery mildew was evaluated visually on all individual leaves and scored as percentage of area affected (Garibaldi et al. 1994; McGrath and Shiskoff 1996).

Preparation of samples for the microscopic examination

For microscopic investigations, plants which inoculated with the powdery mildews were treated with oils 0.5% and 0.5% Tween 20 (control). Samples were harvested two, three, four, five and six days after the third spray. The detached leaves were immersed in clearing solution (0.15% trichloroacetic acid in ethanol: chloroform 4:1) for 24 hours (Hückelhoven at al. 1999). Samples were kept in 50% glycerol before the microscopic examination. The samples were stained with

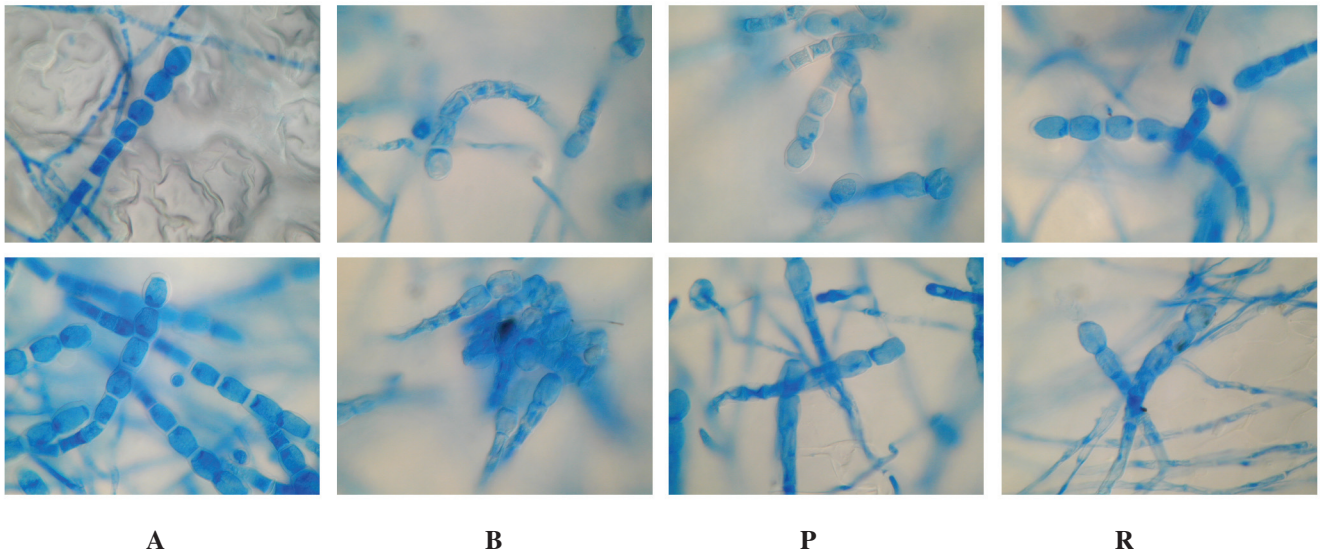


Figure 4. *Podoshaera xanthii* on cucumber sprayed with oils. A: control leaves were sprayed with water + Tween 20, B: black seed oil, P: parafine oil and R: repce oil. Uper row: cucumber sprayed with oils 2 days after inoculation (DAI) and samples were harvested 3 days after the third spray. Down row: cucumber sprayed with oils 3 DAI and samples were harvested 4 days after the third spray. Samples visualized using an Olympus BX 51 microscope with a magnification of 400.

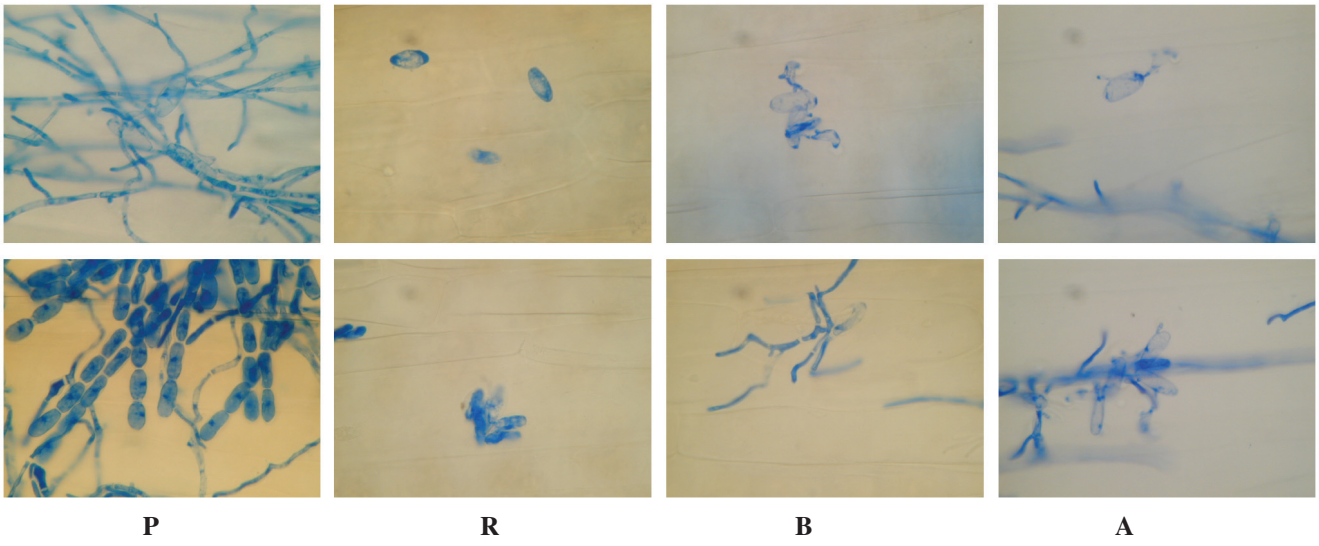
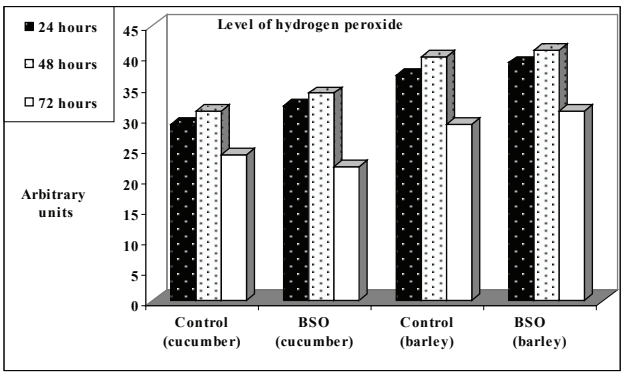


Figure 5. *Blumeria graminis* f. sp. *hordei* on barley sprayed with oils. A: control leaves sprayed with water + Tween 20, B: black seed oil, P: parafine oil and R: rapeseed oil. Uper row: barley sprayed with oils 2 DAI and samples were harvested 3 days after the third spray. Down row: barley sprayed with oils 3 DAI and samples were harvested 4 days after the third spray. Samples visualized using an Olympus BX 51 microscope with a magnification of 400.

blue ink (Pelikan) for one minute, washed in water and put on glass slides to examine under the microscope.

Histo-chemical analysis of $O_2^{\cdot -}$ and H_2O_2

For histo-chemical detection of H_2O_2, Leaves were infiltrated with 0.1% 3, 3-diaminobenzidine (DAB) in 10 mM Tris buffer (pH 7.8). Samples were incubated under daylight for two hours after the vacuum infiltration. After the staining, DAB-treated samples were incubated under daylight for 20 min and subsequently cleared in 0.15% trichloroacetic acid (wt/vol) in ethanol: chloroform 4:1 (vol/vol). The solution was exchanged once during the next 48 h of incubation (Hückelhoven et al. 1999). Subsequently, leaves were stored in 50% glycerol prior to microscopic evaluation.



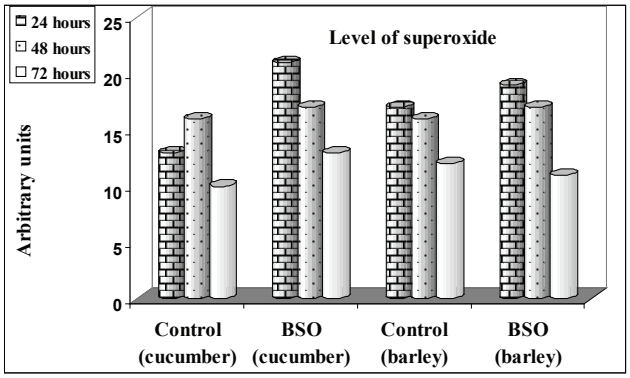


Figure 6. Levels of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) in inoculated cucumber and barley leaves with powdery mildew pathogens and sprayed with oils 3 days after inoculation (3 DAI) three times. Control: leaves were sprayed with water + Tween 20, BSO: leaves were sprayed with black seed oil. The levels of H_2O_2 and $O_2^{\cdot-}$ were measured 24, 48 and 72 hours after the third spray with oil. Means of three independent experiments are shown.

Histo-chemical staining for $O_2^{\cdot-}$ production in leaf tissue was based on the ability of $O_2^{\cdot-}$ to reduce nitro blue tetrazolium (NBT). $O_2^{\cdot-}$ was visualised as a purple coloration of NBT. Leaves discs (2 cm in diameter) were vacuum infiltrated or injected (Hagborg 1970) with 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 w/v % NBT (Sigma–Aldrich, Steinheim, Germany) according to Ádám et al., (1989). NBT-treated samples were cleared as mentioned above.

Discoloration of leaf discs resulted by DAB and NBT staining was quantified using a ChemiImager 4000 digital imaging system (Alpha Innotech Corp., San Leandro, USA). Samples were harvested 24, 48 and 72 hours after the third spray.

Biochemical assays of antioxidant enzymes

Cucumber and barley were sprayed with BSO three days after inoculation with powdery mildew pathogens. Samples were harvested 24, 48 and 72 hours after the third spray. For enzyme assays, 0.5 g leaf material was homogenized at 0-4°C in 3 ml of 50 mM TRIS buffer (pH 7.8), containing 1 mM EDTA-Na_2 and 7.5% polyvinylpyrrolidone. The homogenates were centrifuged (12000 rpm, 20 min, 4°C), and the total soluble enzyme activities were measured spectrophotometrically in the supernatant. All the measurements were carried out at 25°C, using the model UV-160A spectrophotometer (Shimadzu, Japan).

Activities of catalase (CAT), dehaydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione-s-transferase (GST) and ascorbate peroxidase (APX) were determined spectrophotometrically according to Habig et al. 1974; Halliwell and Foyer (1978); Aebi (1984); Asada (1984); Klapheck et al. 1990 and Miyake and Asada (1992).

Results

Effect of oils on powdery mildew symptoms

Powdery mildews symptoms were strongly inhibited after treatment with oils 2 days after inoculation. Symptoms were photographed 2 days after the third spray of cucumber and barley respectively (Fig. 1).

Inhibitory effect of plant oils on spore germination and disease severity

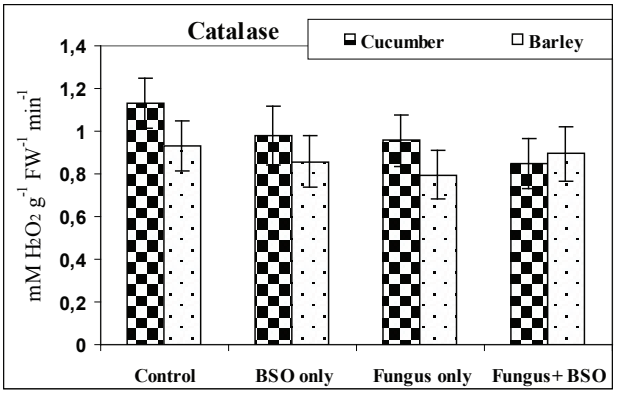
Rate of spore germination was determined on the artificial cellophane media. Figure 2 shows the percentage of germinated conidia of cucumber and barley powdery mildew fungus after treatment with oils. BSO strongly inhibited spores germination compared to other oils (Fig. 2). However, the other two oils showed significant inhibition of the spore germination as compared with the control.

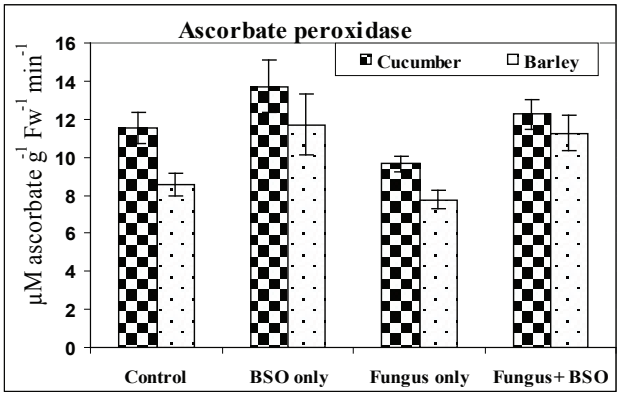
Disease severity (% of infected leaf area/leaf area) of cucumber powdery mildew (*Podoshaera xanthii*) was reduced from 52% (control) to 7.7, 18.6 and 20% on leaves which sprayed with BSO, rapeseed and parafine oils respectively. Disease severity of barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) was reduced from 63.4% (control) to 9.4, 16 and 16.4 % on leaves which sprayed with BSO, rapeseed and parafine oils were respectively (Fig. 3).

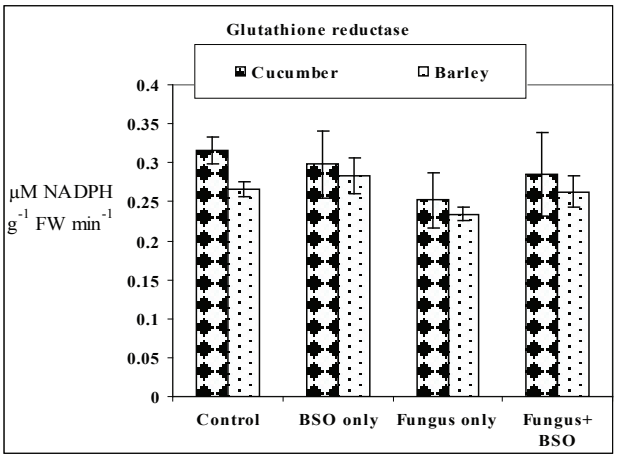
All the three oils were effective in reducing the disease severity of cucumber and barley powdery mildews. When leaves were treated with different concentrations of oils (0, 0.1, 0.30 and 0.50%), 0.5% concentration was the best in reducing the disease severity from 88-90% to 6-16% (Fig. 3).

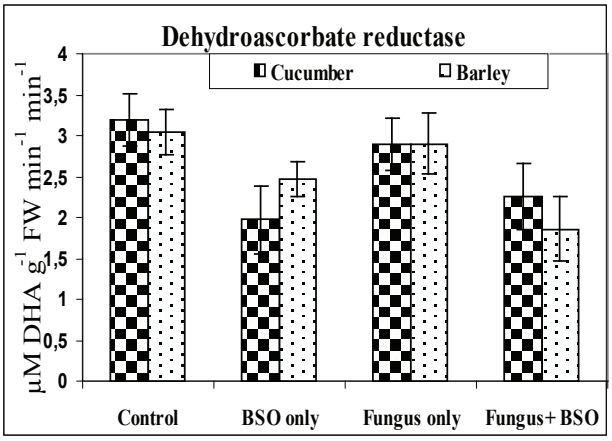
Microscopic examination of cucumber and barley powdery mildew pathogens

Growth of fungal mycelium and spore germination were inhibited by BSO as compared to other oils when the powdery mildew infected cucumber leaves were treated 2 and 3 days after inoculation (Fig. 4). Samples were harvested 3 and 4 days after the third spray (two days in between). Similar results were obtained on barley leaves treated with plant oils three times every 2 days. Samples were harvested 3 and 4 days









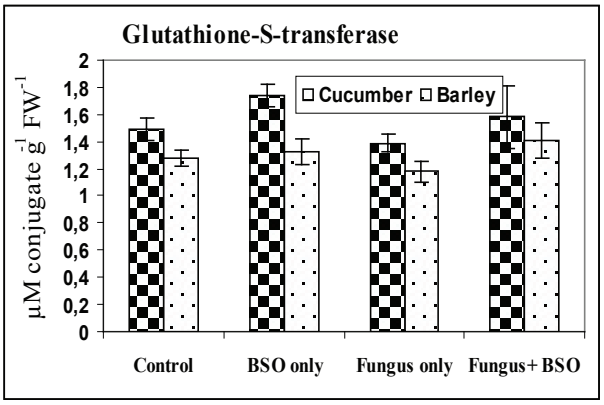


Figure 7. Activities of CAT, DHAR, APX, GST and GR in cucumber and barley leaves which sprayed with black seed oil (BSO) 3 days after infected with powdery mildew pathogens. Enzymes assays were determined 72 hours after the third spray. Means of three independent experiments are shown. Error bars present ± SD.

after the third spray (Fig. 5). Interestingly enough that, BSO was the strongest oil to inhibit the spore germination and the hayphae appeared to be disintegrated (Fig. 4 and 5).

Hydrogen peroxide and superoxide accumulation

H_2O_2 levels were detected in cucumber and barley leaves treated with BSO three times after inoculation with powdery mildew using DAB staining (brown discoloration). BSO treatment increased slightly the level of H_2O_2 only in cucumber as compared to the control which treated only with water + Tween 20 at 24 and 48 hours after the third spray with oil (Fig. 6).

Levels of $O_2^{\cdot-}$ were detected also in powdery mildew inoculated cucumber and barley leaves using NBT staining (blue discoloration). Data indicated that BSO treatment increased the level of superoxide significantly at 24 hours of treatment however BSO did not change the level of $O_2^{\cdot-}$ significantly after 48 or 72 hours of treatment as compared to the control which treated only with water + Tween 20 (Fig. 6).

Activities of antioxidant enzymes

Activities of catalse (CAT), ascorbate peroxidase (APX), glutathione-S-transferase (GST), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) were determined

spectrophotometrically in cucumber and barley leaves which were sprayed with BSO 3 days after the infection with the powdery mildew pathogens. Activities of these enzymes did not change when samples were taken 24 and 48 after the third spary as compared to the control leaves which sprayed only with Tween 20 or infected with the powdery mildew and sprayed with Tween 20 (Fig. 7).

Interestingly, when samples were taken after 72 hours after the third spray, activities of APX and GST increased but activity DHAR was significantly decreased either in cucumber or barley treated only with BSO or infected + BSO treated. However activities of CAT and GR sometimes increased and decreased either in cucumber or barley (Fig. 7).

Discussion

Black seed oil (BSO) from *Nigella sativa* was the most effective in reducing the disease severity and spore germination of cucumber powdery mildew (*Podosphaera xanthii*) and barley (*Blumeria graminis* f.sp. *hordei*). Rapeseed and parafine oils were also effective as compared to control treatment. The antimicrobial activity of BSO has been demonstrated by several researchers (Agarwal et al. 1979; Salem and Hossain 2000; Ali and Blunden 2003) related to human diseases. BSO can also protect plants from pathogens on Arab lands (Rathee at al. 1982; Rahhal 1997; Farid et al. 2000). BSO was very effective against the powdery mildew fungus in squash (Farid et al. 2000).

When leaves were sprayed with 0.05% emulsified oils, powdery mildews were reduced to negligible level. The advantages of using BSO, rapeseed and parafine oils to control powdery mildews are being not expensive, easy to prepare, effective with low concentration and friendly to the environment and human health. Furthermore it can be used for large-scale application to control powdery mildews and it can be ideal for use in organic farming.

By using the toxicity test and the microscopic examination one can clarify that BSO has strong toxic effect against the hyphal growth and spore germination as well as rapeseed and parafine oils. Conidia were damaged dramatically as a result of oils treatments and development of germ tubes was inhibited as compared to control. However, BSO was always very effective as compared with other oils. These results supported by similar results when tomato treated with sunflower oil after inoculation with *Oidium neolycopersici* (Ko et al. 2003), or machine oil which inhibited the conidia germination and become deformed and hyphae deformation (Ohtsuka and Nakazawa 1991; Ohtsuka et al. 1991).

BSO increased slightly the levels of H_2O_2 and $O_2^{\cdot-}$ as well as activities of APX and GST. It's decreased significantly activity of DHAR; however activities of CAT and GR sometimes increased and decreased either in cucumber or barley infected with powdery mildews. The increased levels of H_2O_2 and $O_2^{\cdot-}$ (reactive oxygen species = ROS) could inhibit the spore germination and the hyphal growth of powdery mildew pathogens (Hafez and Kiraly 2003; Hafez 2005). The decreased level of antioxidant enzymes such as DHAR and CAT and GR (sometimes) could be a result of the elevated levels of ROS (H_2O_2 and $O_2^{\cdot-}$). The increased level of antioxidants such as APX and GST as well as CAT and GR (sometimes) protected the cucumber and barley plants (host cells) from the harmful effect of H_2O_2 and $O_2^{\cdot-}$, because the action of the antioxidants is the neutralization of ROS (Hafez and Kiraly 2003; Hafez 2005; Kiraly et al. 2008).

It would seem that, BSO has strong direct inhibitory effect because it contains 36-38% fixed oil, with proteins, alkaloids, saponins, and essential oils making up rest of the composition (Burtis and Bucar 2000). BSO has been reported to possess antimicrobial activity (Morsi 2000), antioxidant activity (Burtis and Bucar 2000) and antitumor activity (Worthen et al. 1998).

The obtained results can be supported and explained by Burtis and Bucar (2000) who made characterization of BSO composition by gas chromatography-mass spectrometry analysis and revealed the presence of a variety of compounds possessing antimicrobial properties, including carvacrol (Ultee et al. 2002), thymol (Rasooli and Mirmostafa 2003), thymohydroquinone (El-Fatatry 1975), thymoquinone (Burtis and Bucar 2000; Ali and Blunden 2003), limonene, carvone (Oumzil et al. 2002), p-cymene and c-terpinene (Gulluce et al. 2003).

One can conclude that, BSO and other oils strongly protected cucumber and barley plants against the powdery mildews through two mechanisms: the major mechanism is the inhibition of conidial germination and suppression of the mycelial growth of the pathogens and the partial mechanism is the activation of the host defence (induced resistance). The author recommends to give more attention to BSO and other oils for integrated pest management.

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