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Maximizing the efficacy of *Trichoderma* to control *Cephalosporium maydis*, causing maize late wilt disease, using freshwater microalgae extracts

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Abstract

The main goal of this study was enhancing the biocontrol activity of *Trichoderma* spp. (*T. harzianum*, *T. koningii*, *T. viride*, and *T. virens*) against *Cephalosporium maydis*, the cause of late wilt disease in maize. Five isolates of *C. maydis* were isolated from diseased maize plants, showing late wilt symptoms, and were collected from infected maize fields in Gharbia Governorate, Egypt. Pathogenicity test revealed that all *C. maydis* isolates were able to attack maize plants (cv. Baladi), which cause late wilt disease. Isolate 3 (Cm3) was the most virulent of them. In in vitro experiments, vegetative growth of the mycelium of *C. maydis* was highly inhibited after opposite sides' treatment by *Trichoderma* species on Potato Dextrose Agar plates amended with *Chlorella vulgaris* extracts (cool and hot extracts) than unamended one. Formulation of *C. vulgaris* extracts and *Trichoderma* spp. were prepared. The formulations maintained the capacity of *Trichoderma* spp. to inhibit growth of the pathogen for up to 1 year when stored at both room temperature or at 7 °C. These formulations (3-day-old) were examined for biological control activities against late wilt disease of maize. Under greenhouse and field conditions, all treatments reduced late wilt incidence compared to the untreated control. Treatments involved *Trichoderma* spp., and *C. vulgaris* extracts were more effective than that used individually. Both of the *C. vulgaris* extracts, with each of *T. virens* and *T. koningii*, were the most effective treatments in this respect. Under greenhouse conditions, formulation treatments (*C. vulgaris* extracts and *Trichoderma* spp.) significantly increase the plant growth of maize plants, i.e., plant height and plant dry weight as compared to the non-treated control either in infested or in un-infested soil with *C. maydis*. Under field conditions, these formulations increased the grain yield as well as ear parameters as compared with either *C. vulgaris* extracts or *Trichoderma* spp. alone as well as non-treated control. This study suggests that the efficacy of *Trichoderma* spp. was enhanced with *C. vulgaris* extracts and these formulations can be developed as bio-fungicides for minimizing the late wilt disease caused by *C. maydis* in maize.

Keywords: *Chlorella vulgaris* extract, Late wilt disease, Maize plants, *Trichoderma* spp., Biological control

Background

Maize, *Zea mays* L., is one of the most important cereal crops worldwide. In Egypt, the cultivated maize area reached about 88,000 ha that yielded almost 7.2 million metric tons of grains (Anonymous 2017). Black bundle disease or late wilt, caused by *Cephalosporium maydis*, is one of the main economical and distributed maize diseases in Egypt (Samra et al. 1963). This disease appears during tasseling as a rapid wilting of the lower leaves and develops to hollow and shrunken stalks with a dark yellow-to-brown or black-stained pith (El-Shafey and Claflin 1999). The pathogen is a soil-borne vascular wilt disease that enters tissue of the root and colonizes the xylem (Sabet et al. 1970). Less commonly, this pathogen can be seed-borne (E1-Shafey et al. 1976) and may irregularly cause decay of seed or pre-emergence damping-off under heavy inoculum pressure (Sabet et al. 1970). This fungus duplicates asexually and has not been in perfect stage (Saleh and Leslie 2004). Large economic

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losses have been reported in Egypt by late wilt disease. In susceptible varieties, the disease affected 70% of the plants decreasing the grain yield by 40% (Labib et al. 1975). Breeding of resistant varieties of maize is the most effective method for controlling this disease (El-Shafey et al. 1988). Various bacteria and actinomycetes have been evaluated as biocontrol agents against late wilt disease (El-Mehalowy et al. 2004 and Ashour et al. 2013). Little information has been cited in the literature on the efficiency of *Trichoderma* spp. against late wilt disease. *Trichoderma* spp. isolated from Egyptian soil were used as a biocontrol for *Colletotrichum dracaenophilum* and *Fusarium proliferatum*, based on results of laboratory trials (Morsy and Elshahawy 2016 and Elshahawy et al. 2017a). It reduced disease caused by the soil-borne fungus *Stromatinia cepivora* (Berk.) and induced plant resistance in onion plants when applied to soil (Elshahawy et al. 2017b).

The microalgae, *Chlorella vulgaris* known as freshwater algae, is one of the most remarkable green microalgae. There are several applications and potential benefits of this microalga such as biofuels, human nutrition, animal feed, wastewater treatment, and agrochemical applications (Safi et al. 2014). *C. vulgaris* contains high amounts of micro- and macronutrients, proteins, and carbohydrates (Wake et al. 1992). It is used as bio-fertilizer and soil conditioner in agriculture systems (Song et al. 2005). Algal extract can be partially substituting micronutrient foliar fertilizers and best to be complementary portion of the spray solution (Shabaan 2010). Soil fertility can be improved by entrapping some rhizosphere bacteria with *Chlorella* (Raposo and Morais 2011). Newly, the consortium of *C. vulgaris*, *Azotobacter* sp., and *Anabaena variabilis* was found to increase germination and plant growth of rice, and it is suggested as a bio-fertilizer and a bio-stimulator for crops as reported by Zayadan et al. (2014).

The present study was conducted to evaluate the efficiency of *Trichoderma* species either alone or mixed with the *C. vulgaris* extracts on the incidence of maize with late wilt under greenhouse and field conditions.

Materials and methods

Experimental site

This study was carried out at the Agriculture and Biological Division, National Research Centre (NRC), as well as within a disease nursery field located at Gharbia Governorate, Egypt, during the 2016 growing season.

Freshwater microalgae, *Chlorella vulgaris*, and preparation of extracts

C. vulgaris was isolated from freshwater Nile River at Cairo Governorate, Egypt (El-Sayed et al. 2001). This strain was massively produced at Algal Biotechnology Unit, National Research Centre, Giza, Egypt. The cultivation was performed, using a 1200-l open-plate photobioreactor. Microalgae nutrition was performed as described by El-Sayed et al. (2015). Grown culture was concentrated and dewatered by gravity. Purification of the obtained biomass was performed by a series of precipitation by cooling centrifuge and washing it using tap water. This procedure was repeated several times to remove any excess of nutrients and mineral elements. The obtained biomass was dried at 45 °C within a circulated oven and then ground to a fine powder (Hassan et al. 2015).

Hot (at 70 °C) and cool water extracts were produced by soaking 10% of microalgae biomass with distilled water and solicited using ultrasonic homogenizer. After homogenization, the extracted materials were obtained by filtration through filter paper (Whatman no. 1). The extracts were freeze-dried and sieved in a refrigerator until used. Total sugars were determined according to Dubois et al. (1956). Polysaccharides were determined in extracts. Firstly, freeze-dried extracts were sequentially treated by petroleum ether and chloroform to remove oiled materials. Absolute ethanol was used to precipitate polysaccharides. Forty milliliters of absolute ethanol was added gradually to 10 ml of water extracts (1:20 *w*/*v*). The mixtures were left overnight into the refrigerator and then centrifuged (5500 rpm for 10 min). The precipitated polysaccharides were dried using a freeze drier and determined by gas-liquid chromatography (GLC).

Trichoderma species

Four *Trichoderma* species, viz., *T. harzianum*, *T. koningii*, *T. viride*, and *T. virens*, were obtained from Plant Pathology Department, NRC, Egypt. The *Trichoderma* species were isolated from Egyptian soil, identified, and evaluated for their efficiency in previous study (Elshahawy et al. 2016).

C. maydis isolates

Maize plant samples, showing typical late wilt symptoms, were collected from naturally infected fields located at Gharbia Governorate, Egypt. Isolation of *C. maydis* was carried out according to Samra et al. (1963). Stems of diseased maize plants were cut into small pieces, and the surface was disinfected with 0.5% sodium hypochlorite for 3 min and then washed thoroughly with sterilized water. The disinfected stem pieces were dried between folds of sterile filter papers, then plated onto potato dextrose agar (PDA) medium supplemented with 0.2% yeast extract and incubated at 28 ± 2 °C for 72 h. Hyphal tip isolation technique was employed to obtain the fungus isolation in pure cultures. *C. maydis* was identified according to morphological and cultural features using the descriptions of Samra et al. (1963) and

Ainsworth and James (1971). Five isolates of *C. maydis* were obtained from diseased maize plants and kept at 4 °C for further studies.

Inoculum preparation and determination of pathogenicity

The isolates of *C. maydis* were grown into 250 ml potato dextrose broth medium supplemented with 0.2% yeast extract in 500 ml Erlenmeyer flasks. After sterilization, flasks were inoculated with each of the different isolates of *C. maydis* and then incubated at 28 ± 2 °C for 2 weeks. The flasks were thoroughly shaken, and about 20 ml of the suspension was poured into 1-l glass bottles containing wet autoclaved crushed grain sorghum up to two thirds of its capacity. The inoculated glass bottles were then kept at 28 ± 2 °C for 4 weeks. Pathogenicity test of the obtained isolates of *C. maydis* was conducted on a susceptible maize cultivar Baladi. Disinfested grain seeds were planted in pots (30 cm in diameter) containing autoclaved clay loam soil (6 kg/pot), infested with the inoculum of different isolates. Seed disinfestations were carried out by soaking seeds in 5% sodium hypochlorite solution for 3 min and rinsed in sterile water. Pots and soil were treated 2 weeks before planting by autoclaving the soil and soaking the pots in 7% formalin solution for 3–5 min. Soil infestation was carried out 7 days before planting by mixing 180 g of inoculum to the soil in every pot and mixed thoroughly to ensure equal distribution of fungal propagates, followed by irrigation. Each pot was seeded with eight grain seeds of the Baladi cv., and plants were thinned to three plants per pot. Six pots were used for each isolate, and a non-inoculated treatment was used as control. Nitrogen fertilizer in the form of urea (46% N) was added at 500 mg N/kg soil, 30 days after planting, and plants were irrigated when necessary. Percentage of dead plants due to late wilt infection was calculated 80 days after planting. Disease symptoms began to appear approximately 60 days after sowing. Pots were examined at weekly intervals thereafter and symptomatic plants removed when they were identified. Fungal isolates were recovered from internodes of symptomatic plants to demonstrate Koch's postulates. Among the tested isolates, the highest aggressive isolate was selected and used throughout the present study. The maize plants were harvested at 80-day age by mulching the plants from the pots. The length of plants and their dry weight were determined. The harvested plants were dried at 70 °C till constant weight, and the dry weight per plant was recorded.

Laboratory experiments

Antagonistic activity tests

Testing the antagonistic activities of *Trichoderma* spp. which uses either alone or in combination with *C. vulgaris* extracts against *C. maydis* was carried out. In the case of *Trichoderma* spp. alone, the inhibitor effect of *T. harzianum*, *T. koningii*, *T. viride*, and *T. virens* against the growth of the most virulent isolate of *C. maydis* (isolate Cm3) was studied, using the method described by Bell et al. (1982). Petri plate containing PDA medium supplemented with 0.2% yeast extract was inoculated on one side with a 5-mm mycelial disc from a 7-day-old culture of the tested *Trichoderma* spp. The opposite side was inoculated with a disc of *C. maydis*, and the plates were incubated at 28 ± 2 °C. Plates inoculated with a disc of *C. maydis* alone were used as control. Four replicate plates were made for each test fungus as well as for the control. Colony radius of *C. maydis* was recorded when the control plates reached full growth. On the other hand, the effect of *C. vulgaris* water extracts on the antagonistic activity of *Trichoderma* spp. against *C. maydis* was carried out, using PDA plates amended with each of cool or hot extracts. Ten milliliters of each extract was filtered through a sterile 0.22-μm Millipore filter directly into 190 ml molten PDA. The medium was poured into sterile Petri plates and cooled at room temperature. The amended plates were used for dual culture test described before. Plates amended with cool extract, hot extract, and sterile distilled water and inoculated with a disc of *C. maydis* by itself were used as control. Four replicate plates were used for each treatment as well as for controls. Colony radius of *C. maydis* was recorded when the control plates reached full growth. The reduction in the growth of *C. maydis* was calculated, using the following formula:

$$\text{Growth reduction } (\%) = [(C-T)/C] \times 100.$$

where C is the average linear growth of *C. maydis* in control and T is the average linear growth of *C. maydis* in biocontrol agent treatment.

Development of *Trichoderma–C. vulgaris* extract formulation

Trichoderma spp. propagules

Trichoderma harzianum, *T. koningii*, *T. viride*, and *T. virens* were grown on a PDA medium at 25 ± 2 °C for 10 days. Afterwards, the mycelium with the spores was scraped from Petri plates and mixed with sterilized distilled water (20 ml/plate) in a blender. The suspension was adjusted by a hemocytometer slide to 10^8 propagates/ml.

Preparation of C. vulgaris extracts

Each of cool or hot extracts of *C. vulgaris* was prepared individually. Two hundred and fifty milliliters of each extract was filtered through a sterile 0.22-μm Millipore filter directly into a 500-ml sterile conical flask.

Incorporation of Trichoderma spp. to C. vulgaris extracts

Propagule suspension (10^8 propagates/ml) of each of *Trichoderma* spp. were individually incorporated into sterilized *C. vulgaris* extracts under aseptic conditions at the rate of 10 ml of suspension per 90 ml extract and thoroughly shacked on a rotatory shaker at 70 rpm for 6 h. Each *Trichoderma–C. vulgaris* extract was first stored at room temperature for 3 days to increase the initial population of *Trichoderma* spp., and then, they were applied.

Population dynamics of Trichoderma spp. on C. vulgaris extracts

The viability of *Trichoderma* spp. in *C. vulgaris* extracts was determined at 3, 60, 120, 180, 240, 300, and 360 days after storage (DAS) of room temperature (27 ± 2 °C). For the study of the potentiality of 7 °C storage conditions on the viability of the *Trichoderma* spp. in *C. vulgaris* extracts, they were first stored at room temperature for 3 days to increase the initial population of *Trichoderma* spp. Initial determination of population of *Trichoderma* spp. was made at 3 DAS at room temperature, and later samples were made at 60, 120, 180, 240, 300, and 360 DAS at 7 °C. Serial dilutions of formulation samples were used to determine the number of *Trichoderma* spp. propagules found on *C. vulgaris* extracts by the plate count technique using selective media (Johnson et al. 1960). Thus, the blended 1 ml of formulation was transferred to bottles containing 99 ml of sterilized distilled water under aseptic conditions. The bottles were shaken using a mechanical shaker for 15 min. Serial dilutions of fresh suspension were prepared for each *Trichoderma* spp. in *C. vulgaris* extract sample under sterile conditions. A portion of 1.0 ml formulation suspension from the dilution 10^{-4} was transferred to four sterile Petri plates. Rose Bengal streptomycin-selective medium was used for growing *Trichoderma* spp. colonies after 4 days of incubation at 25 ± 2 °C (Metcalf 1997). This medium consisted of 2.0 g of $(NH_4)_2SO_4$, 4.0 g of KH_2PO_4, 6.0 g of Na_2HPO_4, 0.2 g of $Fe{\cdot}SO_47H_2O$, 1 mg of $CaCl_2$, 10 μg of H_3BO_3, 10 μg of $MnSO_4$, 70 μg of Zn SO_4, 1 l of distilled water, 20 g agar, and 5 g of cellulose powder (Sigma), adjusted to pH 4.0 before autoclaving. After the medium cooled to 70 °C, 0.05 g of streptomycin sulfate and 0.016 g of rose Bengal were added.

Greenhouse experiments

A pot experiment was conducted to evaluate the influence of *Trichoderma* spp. treatments alone or formulated on *C. vulgaris* extracts on the incidence of maize late wilt as well as on growth parameters of maize plant in soil infected and non-infected with late wilt pathogen. The experiment was conducted in the summer season of 2016 at the greenhouse of Plant Nutrition Department, Agriculture and Biological Division, National Research Centre, Egypt. The experiment was carried out in a randomized complete block design with four replicates. The most virulent isolate of *C. maydis* (isolate Cm3) was used. Seed disinfections were carried out by soaking seeds in 5% sodium hypochlorite solution for 3 min, rinsed in sterile water. Pots (30 cm in diameter) and soil were treated 2 weeks before planting by autoclaving the soil and soaking the pots in 7% formalin solution for 3–5 min. Soil infestation was carried out 7 days before planting by mixing 180 g of *C. maydis* inoculum to the soil in every pot (6 kg soil/pot), followed by irrigation. Disinfected maize grains (Baladi cv.) were soaked in each treatment at the rate of 100 ml/100 grain in 250-ml Erlenmeyer flasks. Control of grains was soaked in sterile distilled water only. Few drops of Tween-80 were added to improve adhesive. Flasks were incubated at 25 °C on a rotary shaker at 70 rpm for 6 h to allow treatment materials to adhere to seeds. After incubation, excess inoculum was removed and grains were left to air-dry for 30 min at room temperature and then immediately planted in the infected and/or un-infected potted soil (Ashour et al. 2013). Each pot was seeded with eight grain seeds, and the plants were thinned to three plants. The abovementioned treatments were applied to soil in the pots with irrigation water at three equal doses (30 ml per pot) each 10 days. Six pots were used for each treatment as well as control. Nitrogen fertilizer in the form of urea (46% N) was added at the rate of 500 mg N/kg soil, 30 days after planting, and the plants were irrigated when necessary.

The following treatments were used in soil infected and non-infected with late wilt pathogen: (1): *T. harzianum* (10×10^4 propagates/ml sterile distilled water), (2): *T. koningii* (10×10^4 propagates/ml sterile distilled water), (3): *T. viride* (10×10^4 propagates/ml sterile distilled water), (4): *T. virens* (10×10^4 propagates/ml sterile distilled water), (5): *T. harzianum* (10×10^4 propagates/ml cool water extract of *C. vulgaris*), (6): *T. koningii* (10×10^4 propagates/ml cool water extract of *C. vulgaris*), (7): *T. viride* (10×10^4 propagates/ml cool water extract of *C. vulgaris*), (8): *T. virens* (10×10^4 propagates/ml cool water extract of *C. vulgaris*), (9): *T. harzianum* (10×10^4 propagates/ml hot water extract of *C. vulgaris*), (10): *T. koningii* (10×10^4 propagates/ml hot water extract of *C. vulgaris*), (11): *T. viride* (10×10^4 propagates/ml hot water extract of *C. vulgaris*), (12): *T. virens* (10×10^4 propagates/ml hot water extract of *C. vulgaris*), (13): Cooled water extract of *C. vulgaris*, (14): Heat water extract of *C. vulgaris*, (15): Control.

Percentage of dead plants due to late wilt infection was recorded 80 days after planting. Vegetative growth parameters, i.e., plant height and dry weight, were also recorded as previously described.

Field experiments

The effect of *Trichoderma* spp. treatments alone or formulated on *C. vulgaris* extracts on the incidence of maize late wilt as well as on yield of maize plant was studied under field conditions in a disease nursery at Gemmiza Research Station, Plant Pathology Research Institute, Agriculture Research Center, Gharbia Governorate, Egypt, during the 2016 growing season. This nursery was infested artificially with the four clonal lineages of *C. maydis* found in Egypt that causes late wilt of maize and commonly used in Egyptian maize breeding programs (Zeller et al. 2002). Maize grains cv. Baladi were used in this study. The abovementioned treatments in greenhouse were involved in field experiments. Disinfected maize grains (Baladi cv.) were soaked in each treatment at the rate of 100 ml/100 grain. Control grains were soaked in sterile distilled water only. Randomized complete block arrangement in three replicate plots was used. Each replicate included three ridges of 4.5-m length and 0.7-m width for each ridge, i.e., the experimental plot area was 3.15 m^2. Thirteen maize plants for each treatment were used in each replicate. Grains were sown in holes (five holes/ridge with three grains/hole); thereafter, they were thinned to one plant/hole. The abovementioned treatments were also applied before irrigation with water at three equal doses (10 ml per hole) each 15 days. Irrigation, recommended fertilizer levels, and agronomical practices were used as usual. Disease incidence of late wilt as infection percentage was recorded 110 days after sowing. Quantitative maize yield and qualitative maize yield, i.e., ear length, ear diameter, no. of rows per ear, no. of kernels per row, no. of kernels per ear, and 100-kernel weight, were evaluated during harvest period.

Statistical analysis

Statistical analysis of data was conducted, using SPSS software version 14.0. Percent data of disease incidence were statistically analyzed after arcsine square root transformation; however, untransformed data are presented. Analysis of variance was determined, and the mean values were compared by Duncan's multiple range test at $P < 0.05$.

Results and discussion

Chemical analysis of *Chlorella* extract

Chlorella as the freshwater microalgae is considered the most useful green algae (Liu et al. 2016). It contains lipopolysaccharides which differ from Gram-negative bacteria because chlorella has no endotoxins (Stewart et al. 2006). Dried biomass total sugars of *C. vulgaris* represented 9.7% + 0.12. Out of this content, soluble sugar content, which is determined by weight, varied between the two fractions (cold and hot extracts). Mostly, hot extract represented the maximum figure of total sugars (16.42 and 12.31%) of total carbohydrates. GLC analysis of each fraction is listed in Table 1. The most abundant sugars are galactose, mannose, rhamnose, and glucose that reached more than 10% of total carbohydrates. Concerning such chemical structure, growth conditions (outdoor mass production) markedly affected it to form a rigid cell wall. This is in agreement with Cheng et al. (2011), who described that the chemical composition of the cell wall in *Chlorella variabilis* NC64A was impacted by cultivation conditions such as uronic acid, neutral sugar, and amino sugar in the cell wall when cultivated in diverse sources and concentrations of nitrogen. Unbending cell walls of *Chlorella* species contain mannose as a major sugar component. Numerous polysaccharides contained phosphate, carboxylic, and/or ester sulfuric groups in the molecular structure (Nelson and Cox 2008). These polysaccharides, in a pure form, are presently the most commercial protectors for plants against pathogens (Stadnik and Freitas 2014).

C. maydis isolation

Five isolates of *C. maydis* obtained from infected maize plants were studied on a susceptible cultivar Baladi. For recording infection percentage by late wilt, typical disease symptoms formerly described by Samra et al. (1963) were observed on infected plants. Koch's postulates were demonstrated for all *C. maydis* isolates recovered from infected maize plants in the field. All *C. maydis* isolates were examined for pathogenicity toward maize plants in greenhouse. Results in Table 2 showed that the five isolates were capable of causing late wilt disease and were potentially pathogenic in greenhouse assay system. Non-inoculated plants (control) did not develop late wilt symptoms. Data also showed that the tested *C. maydis* isolates were statistically differed in their aggressiveness toward maize plants where disease percentages varied between 65.0 and 78.2%, at 110 days after sowing. The most virulent isolate was Cm3,

Table 1 Intercellular saccharide content (% dw from total carbohydrates) of *Chlorella vulgaris* cool and hot extract

Saccharide	% content (dry weight from total carbohydrates)	
	Cool	Hot
Galactose	25.5	26.30
Mannose	16.3	11.90
Rhamnose	14.5	18.20
Glucose	13.4	10.60
Arabinose	09.7	10.70
Xylose	06.6	08.90
Fructose	02.9	02.96
Ribose	02.8	02.03

followed by Cm4, while the isolates Cm1, Cm2, and Cm5 were the least ones. These findings are in agreement with those obtained by Ali (2000). García-Carneros et al. (2012) found that the initial incidence of late wilt symptoms in maize plants depends on the isolate of *C. maydis* and on the maize variety and the final severity of the aboveground symptoms only depends on the fungal isolate. The pathogenic differences among the tested isolates may be due to the genetic diversity among them. Zeller et al. (2002) found that the four phylogenetic lineages of *C. maydis* differed in their virulence and competitiveness toward maize plants grown under greenhouse conditions. A highly negative correlation was observed among infection percentages incited by *C. maydis* isolates and each of plant height and dry weight of maize plants after 110 days. These results are in harmony with the findings of Alhanshoul (2015) who reported that infection with *C. maydis* isolates slightly reduced seed germination, plant height, and dry weight of plants.

Antagonistic activity of *Trichoderma* spp. and *C. vulgaris* extracts against *C. maydis*

Four *Trichoderma* species were tested alone or in combination with *C. vulgaris* extracts for antagonistic activity against *C. maydis*, using the dual culture technique (Table 3). All *Trichoderma* species treatments inhibited the growth of *C. maydis* in dual culture compared to control. Data indicated that *T. harzianum*, *T. viride*, *T.virens*, and *T. koningii* reduced the growth of *C. maydis* by 63.3, 50.0, 75.6, and 70.9%, respectively, when used alone, while the reduction attained to 81.1 and 80.0, 61.1 and 60.4, 87.6 and 88.0, and 85.8 and 86.9% when they were used in combination with cool and hot extracts of *C. vulgaris*, respectively. *Trichoderma* species showed rapid growth, outcompeting the pathogen for space and nutrients. After the *Trichoderma* species growth meets the *C. maydis* colony, it would inhibit further growth of the hyphal tips of the pathogen and caused the die back rapidly of fungal colony. *Trichoderma* spp. produce their biocontrol action against fungal phytopathogens either indirectly by competing for nutrients and space or indirectly by mechanisms such as antibiosis and mycoparasitism (Benítez et al. 2004). The additive effect of microalgae extracts to *Trichoderma* species in dual culture may be due to its bio-stimulators and immunity effects. These microalgae produce growth-promoting regulators, vitamins, amino acids, polypeptides, and polymers such as exo-polysaccharides (Singh et al. 2005). Wake et al. (1992) reported that freshwater microalgae as *C. vulgaris* contain high amounts of micro- and macronutrients (metabolites) as proteins and carbohydrates. These bio-fertilizers enhance *Trichoderma* growth and subsequently its antagonistic agent production. On the other hand, the reduction of *C. maydis* growth caused by *C. vulgaris* extracts may be due to the low saprophytic behavior of *C. maydis* (Sabet et al. 1970) that minimizes its growth on PDA amended with algae extracts.

Population dynamics of *Trichoderma* spp. on *C. vulgaris* extracts

The results of the effect of cool and heat extracts of *C. vulgaris*-based liquid formulations on the population dynamics of *Trichoderma* spp. at room temperature indicated that the algae extracts supported the highest population of *Trichoderma* spp. during the DAS sampled

Table 2 Virulence of *Cephalosporium maydis* isolates on maize cv. Baladi under greenhouse conditions

Isolate	Disease incidence (%)	Plant's vigor	
		Plant height (cm)	Plant dry weight (g)
C. maydis (Cm1)	67.2 ± 0.37c	66.2 ± 0.37bc	29.2 ± 0.48b
C. maydis (Cm2)	65.0 ± 1.00d	64.6 ± 0.40c	27.0 ± 0.31c
C. maydis (Cm3)	78.2 ± 0.37a	59.4 ± 0.60d	23.6 ± 0.50d
C. maydis (Cm4)	74.4 ± 1.16b	66.8 ± 0.37b	28.0 ± 0.31bc
C. maydis (Cm5)	67.6 ± 0.60c	66.6 ± 0.24bc	26.8 ± 0.37c
Control	00.0	83.4 ± 1.40a	34.8 ± 0.20a

The presented data are the mean ± standard errors, and the letters show significance at $P \leq 0.05$

Table 3 Antagonistic activity of *Trichoderma* spp. alone or in combination with *Chlorella vulgaris* extract against the linear growth of *Cephalosporium maydis*

Treatment	Linear growth and growth reduction of *C. maydis*	
	Linear growth (cm)	Reduction (%)
T. harzianum (Th)	3.30 ± 0.20d	63.3
T. viride (Tv)	4.50 ± 0.15c	50.0
T. virens (Tvs)	2.20 ± 0.12f	75.6
T. koningii (Tk)	2.62 ± 0.05e	70.9
Cool extract + Th	1.70 ± 0.12g	81.1
Cool extract + Tv	3.50 ± 0.15d	61.1
Cool extract + Tvs	1.12 ± 0.04h	87.6
Cool extract + Tk	1.28 ± 0.03h	85.8
Hot extract + Th	1.80 ± 0.12g	80.0
Hot extract + Tv	3.56 ± 0.16d	60.4
Hot extract + Tvs	1.08 ± 0.04h	88.0
Hot extract + Tk	1.18 ± 0.04h	86.9
Cool extract	7.88 ± 0.08b	12.4
Hot extract	7.64 ± 0.17b	15.1
Control	9.00 ± 0.00a	00.0

The presented data are the mean ± standard errors, and the letters show significance at $P \leq 0.05$

(data not shown). The population of *Trichoderma* spp. found on cool and heat extracts of *C. vulgaris* followed a fluctuating trend with the DAS sampled. The initial population (3 days) of *Trichoderma* spp. was increased at 60 DAS. At 120 DAS, the population recovery in all the *Trichoderma* spp. was significantly increased. Thereafter, during the period of 180 to 300 DAS, the population of *Trichoderma* spp. was declined progressively, and at 360 DAS, a significant reduction of more than four- to ninefold was recorded. The results of the population dynamics of *Trichoderma* spp. in extracts of *C. vulgaris*, when the liquid formulations were stored at 7 °C, showed that there was a slow and progressive decline of the antagonist populations in the bioformulations from 120 DAS to 180, 240, 300, and 360 DAS. Still, the population recovered was much greater as compared to that recorded during the same period under room temperature storage conditions. These formulations maintained the capacity of *Trichoderma* spp. to inhibit growth of the pathogen for up to 1 year when stored at both room temperature or at 7 °C (data not shown).

Efficiency of treatments on maize late wilt disease

After obtaining positive reaction of using microalgae cool or hot water extracts with *Trichoderma* spp. in controlling of *C. maydis*, the experiments were applied under greenhouse and field conditions. This confirms the efficiency of applying *C. vulgaris* extracts as growth promoters and biocontrol agents. Under greenhouse conditions, data presented in Table 4 showed that seed + soil treatment with *Trichoderma* spp. either alone or in combination with *C. vulgaris* extracts significantly reduced the infection percentage with late wilt disease compared to check treatment (73.4% infection) under artificial soil infestation. Treatment with *Trichoderma* spp. formulated on *C. vulgaris* extracts gave the highest effect in reducing infection percentages compared to *Trichoderma* spp. alone. Among the *Trichoderma* spp., *T. virens* formulated on hot extract of *C. vulgaris*, followed by *T. virens* formulated on cool extract and *T. koningii* formulated on cool extract, were the effective treatments in reducing infection percentages, being 72.1, 68.3, and 67.6% reduction in disease incidence, respectively. Other *Trichoderma* spp. treatments used either alone or in combination with *C. vulgaris* extracts showed moderate effect. Treatment with *C. vulgaris* extracts alone showed the lowest effect in reducing the infection percentage with late wilt.

In the field experiment, treatment of seed and soil with *Trichoderma* spp. either alone or in combination with *C. vulgaris* extracts showed significant reduction in maize infection with late wilt compared to check plants (47.6% infection) as presented in Table 4. The combination treatments of *Trichoderma* spp. with *C. vulgaris* extracts caused more reduction in maize late wilt compared to the individual treatments of *Trichoderma* spp. or *C. vulgaris* extracts. However, the combined treatment of *C. vulgaris* extract with *T. virens* and/or *T. koningii* gave the highest reduction in late wilt incidence, being 73.1 and 74.0%, respectively, in comparison to treatment with *T. virens* or *T. koningii* each alone, being 50.0 and 48.3% reduction, respectively. The lowest reduction in disease incidence was recorded by *C. vulgaris* extract treatments only (being 1.7 and 8.0% reduction). The antagonistic effect of *Trichoderma* strains against soil-borne fungi was recently emphasized by Elshahawy et al. (2017b). The reported data describe, for the first time, control of the pathogen, using *Trichoderma* strains in Egypt. The ability of *Trichoderma* strains to inhibit *C. maydis* pathogen is noteworthy since the suppression obtained is a result of seed + soil treatment. Addition of *Trichoderma* strains with *C. vulgaris* extracts increased their effect against *C. maydis*. These results suggest that *C. vulgaris* extracts stimulate the inhibitory activity of *Trichoderma* strains. This may be due to that *C. vulgaris* extracts are considered as absorbed agents into plants for increasing of disease and stress resistance (Abd El-Motty et al. 2010). Amendment of *Trichoderma* strains with *C. vulgaris* extracts could increase the plant protection by supporting the growth of *Trichoderma* strains and stimulating the useful metabolite production which may help antagonistic activity. Degani et al.

Table 4 Effect of *Trichoderma* spp. alone or in combination with *Chlorella vulgaris* extract on the incidence of late wilt of maize grown under greenhouse and field conditions

Treatment	Late wilt incidence (%)	
	Greenhouse experiment	Field experiment
T. harzianum (Th)	45.2 ± 0.86c	31.8 ± 0.48c
T. viride (Tv)	37.6 ± 0.51d	28.6 ± 0.50d
T. virens (Tvs)	33.6 ± 0.24e	23.8 ± 0.37e
T. koningii (Tk)	30.2 ± 0.20f	24.6 ± 0.24e
Cool extract + Th	30.0 ± 0.31f	20.8 ± 0.48f
Cool extract + Tv	27.6 ± 0.24g	16.4 ± 0.40g
Cool extract + Tvs	23.2 ± 0.20h	12.8 ± 0.48h
Cool extract + Tk	23.8 ± 0.37h	12.4 ± 0.40h
Hot extract + Th	27.6 ± 0.24g	17.6 ± 0.40g
Hot extract + Tv	30.0 ± 0.31f	19.6 ± 0.74f
Hot extract + Tvs	21.0 ± 0.31i	12.4 ± 0.40h
Hot extract + Tk	22.4 ± 0.24hi	12.4 ± 0.40h
Cool extract	57.4 ± 0.81b	46.8 ± 0.48a
Hot extract	57.2 ± 0.37b	43.8 ± 0.20b
Control	73.4 ± 1.66a	47.6 ± 0.24a

The presented data are the mean ± standard errors, and the letters show significance at $P \leq 0.05$

(2015) reported that many plant growth promoters such as hormones, auxin (indole-3-acetic acid), and cytokinin (kinetin) were produced by higher levels, when using *C. vulgaris* extracts, suppressing *C. maydis* in culture media and in a detached root assay.

Efficiency of treatments on maize growth in greenhouse and on maize yield in field

Greenhouse experiment

Data presented in Table 5 showed that the seed + soil treatment with *Trichoderma* spp., either alone or in combination with *C. vulgaris* extract, significantly promoted plant growth compared to check treatment, whether grown in infested or un-infested soil. Moreover, *Trichoderma* spp. formulated with *C. vulgaris* extract caused significant increment in maize growth parameters compared to treatment of *Trichoderma* spp. alone. Regarding plant height, data indicated that all treatments significantly increased plant height in either infested or un-infested soil, being 60.4 and 78.41 cm, respectively. Combined treatments of *Trichoderma* spp. and microalgae extracts increased plant height when plants were grown, either in infested or in un-infested soil compared to individual treatments. However, the combined treatment of *T. virens* and *T. koningii* with cool extract of *C. vulgaris* caused the highest plant heights when plants were grown either in infested soil (103.6 and 106.4 cm) or in un-infested soil (119.6 and 118.0 cm) in comparison to the individual treatments. The combination treatments of *T. virens* and *T. koningii* with hot extract of *C. vulgaris*, followed the previous treatments in their effect on plant height, being 102.2 and 102.8 cm for plants grown in infested soil as well as 115.8 and 116.4 cm when grown in un-infested soil, respectively. Other treatments showed moderate effects. In concern to plant dry weight, results presented in Table 4 showed that all treatments recorded significant increment in dry weight of maize plants grown either in infested or in un-infested soil compared to check plants (24.2 and 33.8 g, respectively). Likewise, combined treatments between *Trichoderma* spp. and microalgae extracts significantly increased dry weight of plants in comparable with the individual treatments for plants grown either in infested or in un-infested soil. However, the combined treatments of *T. virens* and *T. koningii* with cool extract of *C. vulgaris* gave the highest dry weight of plants grown either in infested soil (49.4 and 50.6 g, respectively) or in un-infested soil (58.4 and 58.4 g, respectively). The treatment of *Trichoderma* spp. and algae extracts had moderate effect on plant dry weight either grown in infested soil or in un-infested soil. The effect of bio-fertilization, using microalgae extracts, was suggested for increasing the growth parameters of many plants. This is likely due to the biochemical composition of microalgae extracts which are rich in essential nutrients for plant growth as nitrate reductase, nitrogenase, and minerals. The impact of foliar feeding by water extracts of *C. vulgaris* on growth, nutrient levels, and yield

Table 5 Effect of *Trichoderma* spp. alone or in combination with *Chlorella vulgaris* extract on maize plant growth characters under infested and un-infested soil with *Cephalosporium maydis*

Treatment	Plant's vigor			
	Un-infested soil		Infested soil	
	Plant height (cm)	Plant dry weight (g)	Plant height (cm)	Plant dry weight (g)
T. harzianum (Th)	90.4 ± 0.24j	38.4 ± 0.24h	83.6 ± 0.24j	33.4 ± 0.24i
T. viride (Tv)	88.2 ± 0.20k	36.4 ± 0.24i	84.0 ± 0.54j	31.4 ± 0.24j
T. virens (Tvs)	91.6 ± 0.24i	40.4 ± 0.24g	88.4 ± 0.24h	33.8 ± 0.20hi
T. koningii (Tk)	93.4 ± 0.24g	41.4 ± 0.24f	87.4 ± 0.24i	34.2 ± 0.20h
Cool extract + Th	103.4 ± 0.24d	50.2 ± 0.20c	100.6 ± 0.40d	43.4 ± 0.24d
Cool extract + Tv	100.0 ± 0.54f	50.4 ± 0.24c	100.8 ± 0.37d	44.4 ± 0.24c
Cool extract + Tvs	119.6 ± 0.24a	58.4 ± 0.24a	103.6 ± 0.24b	49.4 ± 0.24b
Cool extract + Tk	118.0 ± 0.31b	58.4 ± 0.24a	106.4 ± 0.24a	50.6 ± 0.24a
Hot extract + Th	103.8 ± 0.20d	50.8 ± 0.37c	96.0 ± 0.31f	42.6 ± 0.24e
Hot extract + Tv	101.4 ± 0.40e	50.4 ± 0.24c	97.2 ± 0.20e	43.0 ± 0.31de
Hot extract + Tvs	115.8 ± 0.20c	55.4 ± 0.24b	102.2 ± 0.20c	49.4 ± 0.24b
Hot extract + Tk	116.4 ± 0.24c	55.6 ± 0.24b	102.8 ± 0.20c	49.6 ± 0.24b
Cool extract	93.6 ± 0.24g	44.4 ± 0.24d	88.6 ± 0.24h	35.0 ± 0.44g
Hot extract	92.6 ± 0.24h	43.4 ± 0.24e	90.6 ± 0.24g	37.6 ± 0.24f
Control	78.4 ± 0.24l	33.8 ± 0.37j	60.4 ± 0.24k	24.2 ± 0.20k

The presented data are the mean ± standard errors, and the letters show significance at $P \leq 0.05$

of wheat (*Triticum aestivum* L.) var. Giza 69 was investigated by Shabaan (2010). They reported that 50% (*v*/*v*) microalgae extracts foliar spray, after 25 days of sowing, increased plant growth and grain weight by 140 and 40%, respectively. The present study showed that the addition of *C. vulgaris* water extracts to the culture medium or soil increased the fresh and dry weight of maize seedlings. Abd El-Motty et al. (2010) reported that spraying of 2% algae combined with 0.2% yeast on Keitte mango trees once at full bloom had improved nitrogen, potassium, and boron contents in the leaves. In this respect, Taha and Youssef (2015) reported a significant increase of growth mass as well as content of phosphorous, potassium, and chlorophyll of maize plants grown in soil treated with green microalgae strains of *Chlorella*.

Field experiment

Following the greenhouse experiments, field experiments were the advanced confirmation for the aim of this work. Data in Tables 6 and 7 showed that seed + soil treatment with *Trichoderma* spp. either alone or in combination with *C. vulgaris* extracts significantly improved crop production and ear characters compared to check plants. Moreover, combination of *Trichoderma* spp. with *C. vulgaris* extracts caused markedly an increase in maize yield parameters compared to the individual treatments. All treatments increased the yield of maize plants. The treatment of *T. virens* in combination with cool and hot extracts of *C. vulgaris* caused the highest of ears, weight of grains, and weight of grains/plant, being 4338.0 and 4369.3, 2227.0 and 2229.4, and 348.0 and 352.1, respectively, compared to the control, being 1165.7, 893.1, and 195.0 g. In contrast, plants treated with cool extract of *C. vulgaris* alone produced the lowest yield compared to the other treatments (Table 6).

The same trend was observed in regard to 100-kernel weight (Table 6). The highest weight was recorded in the case of combination treatments of *Trichoderma* spp. with *C. vulgaris* extracts. The lowest 100 grain weight was obtained by the control treatment (28.34 g). Data in Table 6 also indicated insignificant differences among treatments concerning ear parameters of ear diameter, ear length, no. of rows per ear, and no. of kernels per row. All treatments increased ear parameters compared to check plants. Combined treatments of *Trichoderma* spp. with *C. vulgaris* extracts caused the highest ear parameters. Microalgae extracts containing many nutrients as N, P, Ca, K, S, and Mg, as well as some trace elements as Fe, Zn, Mn, Mo, Co, and Cu and some growth regulators, vitamins, and polyamines, were applied to stimulate vegetative growth, nutritional levels, yield, and fruit quality of different orchard as well as vineyards (Abd El-Migeed et al. 2004 and Spinelli et al. 2009).

Conclusions

Trichoderma spp. are one of the proven biological control agents. In the present study, the antagonism of *Trichoderma* strains in combination with *C. vulgaris* extracts increased the efficiency of controlling the maize late wilt disease. Treatments also increased maize plant growth and yield. It is suggested that extracellular

Table 6 Effect of *Trichoderma* spp. alone or in combination with *Chlorella vulgaris* extract on yield of maize plants grown under field conditions

Treatment	Maize plants grown under field conditions		
	Av. weight of ears/plot (g)	Av. weight of grains/plot (g)	Av. weight of grains/plant (g)
T. harzianum (Th)	2780.0 ± 14.4f	1796.7 ± 2.66g	227.0 ± 1.77de
T. viride (Tv)	2716.0 ± 10.5f	1688.6 ± 0.32h	208.0 ± 1.086e
T. virens (Tvs)	3243.3 ± 12.3d	2059.7 ± 3.80c	235.4 ± 1.70cde
T. koningii (Tk)	3068.0 ± 13.0e	2046.9 ± 0.85d	226.8 ± 2.88de
Cool extract + Th	3510.0 ± 8.6c	1934.3 ± 6.28e	310.4 ± 32.67ab
Cool extract + Tv	3495.0 ± 22.9c	1830.4 ± 3.35f	261.3 ± 25.43bcde
Cool extract + Tvs	4338.0 ± 10.3a	2227.0 ± 1.26ab	348.0 ± 38.19a
Cool extract + Tk	4046.0 ± 19.6b	2220.2 ± 0.29b	327.4 ± 37.52ab
Hot extract + Th	3520.0 ± 13.2c	1929.0 ± 3.95e	302.8 ± 34.99abc
Hot extract + Tv	3490.0 ± 21.7c	1827.0 ± 5.59f	296.6 ± 32.12abcd
Hot extract + Tvs	4369.3 ± 27.2a	2229.4 ± 2.57a	352.1 ± 38.86a
Hot extract + Tk	4040.3 ± 5.6b	2224.2 ± 1.62ab	311.6 ± 35.74ab
Cool extract	2003.3 ± 26.0h	1500.6 ± 0.36i	208.0 ± 0.68e
Hot extract	2185.3 ± 14.6g	1503.0 ± 0.61i	205.6 ± 0.16e
Control	1165.7 ± 70.3i	893.1 ± 5.97j	195.0 ± 1.11e

The presented data are the mean ± standard errors, and the letters show significance at $P \leq 0.05$

Table 7 Effect of *Trichoderma* spp. alone or in combination with *Chlorella vulgaris* extract on average parameters of yield component of maize plants grown under field conditions

Treatment	Average parameters of yield component of maize plants grown under field conditions				
	Av. ear length (cm)	Av. ear diameter (cm)	No. of row/ear	No. of kernel/row	100 kernel weight (g)
T. harzianum (Th)	22.59 ± 0.05e	4.11 ± 0.03e	12.8 ± 0.33abc	40.2 ± 0.13g	32.29 ± 0.07fg
T. viride (Tv)	22.31 ± 0.08f	4.26 ± 0.03 cd	12.4 ± 0.26c	40.7 ± 0.15fg	32.14 ± 0.09g
T. virens (Tvs)	22.58 ± 0.10e	4.28 ± 0.03bcd	12.4 ± 0.26c	41.6 ± 0.16e	32.78 ± 0.08defg
T. koningii (Tk)	22.79 ± 0.02cd	4.35 ± 0.01a	13.0 ± 0.33abc	41.0 ± 0.25ef	32.53 ± 0.18efg
Cool extract + Th	22.99 ± 0.01a	4.35 ± 0.01a	13.2 ± 0.32abc	44.6 ± 0.22bc	33.47 ± 0.13bcd
Cool extract + Tv	22.90 ± 0.02abc	4.24 ± 0.01d	13.0 ± 0.33abc	43.5 ± 0.16d	33.32 ± 0.14cde
Cool extract + Tvs	22.85 ± 0.02bc	4.36 ± 0.01a	13.6 ± 0.26a	45.0 ± 0.25ab	33.52 ± 0.17abcd
Cool extract + Tk	22.94 ± 0.02ab	4.36 ± 0.01a	13.4 ± 0.30ab	45.0 ± 0.21ab	33.56 ± 0.15abcd
Hot extract + Th	22.94 ± 0.02ab	4.24 ± 0.01d	13.0 ± 0.33abc	44.7 ± 0.26bc	33.76 ± 0.06abc
Hot extract + Tv	22.85 ± 0.02bc	4.24 ± 0.01d	12.8 ± 0.32abc	44.3 ± 0.15c	33.02 ± 0.09cdef
Hot extract + Tvs	22.93 ± 0.03ab	4.34 ± 0.03ab	13.6 ± 0.26a	45.5 ± 0.22a	34.23 ± 0.01ab
Hot extract + Tk	22.97 ± 0.02ab	4.32 ± 0.02abc	12.6 ± 0.30bc	45.2 ± 0.24ab	34.35 ± 0.12a
Cool extract	22.66 ± 0.03de	4.24 ± 0.01d	12.4 ± 0.26c	40.4 ± 0.16fg	30.19 ± 0.11h
Hot extract	22.78 ± 0.03cd	4.26 ± 0.01cd	12.4 ± 0.26c	40.5 ± 0.16fg	29.66 ± 0.39h
Control	22.37 ± 0.08f	3.86 ± 0.01f	10.4 ± 0.26d	33.6 ± 0.45h	28.34 ± 1.04i

The presented data are the mean ± standard errors, and the letters show significance at $P \leq 0.05$

saccharides content of *C. vulgaris* extracts enhanced the growth and adhesion of *Trichoderma* spp. which promoted plant growth through increasing antifungal activity.

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Authors' contributions
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