Phytoparasitica, September 2014, Volume 42, Issue 4, pp 559-570

The final publication is available at the following Link:

http://link.springer.com/article/10.1007/s12600-014-0394-5

CHEMICAL CONTROL OF MAIZE LATE WILT IN THE FIELD

Ofir Degani[a,b*], Tsafrir Weinberg[c] and Shaul Graph[b]

(The accepted manuscript version)

[a] Tel-Hai College, Upper Galilee, 12210, Israel

[b] Migal – Galilee Research Institute, P.O. Box 831 Kiryat Shmona, 11016, Israel

[c] Galilee Seeds, Research and Development (1989) Ltd., P.O. Box 119, Rosh Pina, 12000, Israel

*Corresponding author:

Ofir Degani, Email: d-ofir@bezeqint.net; ofird@telhai.ac.il

Molecular Phytopathology Lab, Migal – Galilee Research Institute, Southern Industrial Zone, P.O. Box 831, Kiryat Shmona 11016, Israel. Tel.: 972-54-6780114, Fax: 972-4-6817410

Summary. Late wilt, a severe vascular disease of maize caused by the fungus *Harpophora maydis*, is characterized by relatively rapid wilting of maize plants before tasseling and until shortly before maturity. In Israel, the disease has become a major problem in recent years. The pathogen is currently controlled using cultivars of maize having reduced sensitivity. In an earlier work, we modified a molecular method for use as a diagnostic tool to evaluate disease progression in field-infested plants and showed that several fungicides suppressed *H. maydis in vitro*. Here, we examine the effect of different fungicides on disease progression in a contaminated maize field in the spring and summer of 2009 and 2010. The field was watered using a drip irrigation line for each row and the fungicides were injected directly into the drip line. One of the four fungicides tested, Azoxystrobin, was highly effective compared to the control, inhibited the development of wilt symptoms and recovered cob yield by 100%. Although this is the first success in preventing disease symptoms in infested fields in Israel, the Azoxystrobin treatment did not decrease the amounts of pathogen DNA in host tissues or delay its spread. Attempts to reduce concentrations of this fungicide or to apply it by spraying were less effective than the triple full dosage treatment. The presence of the pathogen in the host tissues of the successfully treated plants and its ability to undergo pathogenic variations are increasing the risk of pathogen resistance and the urgent need to develop new ways of controlling late wilt.

Keywords: Azoxystrobin; fungicide; fungus; *Harpophora maydis*; late wilt; maize

INTRODUCTION

Late wilt, or black bundle disease, is a vascular wilt disease of corn caused by the soil-borne and seed-borne fungus, *Harpophora maydis* (Michail et al. 1999; Samra et al. 1966; Gams 1971), with synonyms *Cephalosporium maydis* (Samra, Sabet and Hingorani) and *Acremonium maydis* (El-Shafey and Claflin 1999). The fungus reproduces asexually, and no perfect stage has been identified (Zeller et al. 2000). This disease was first reported as a vascular wilt disease of corn in Egypt in 1960 (Sabet et al. 1961) and is now considered endemic throughout Egypt. Late wilt was also reported in India (Payak et al. 1970), Hungary (Pecsi and Nemeth 1998), Israel (Drori et al. 2012), Spain and Portugal (Molinero-Ruiz et al. 2011). Serious economic losses from late wilt have been reported in Egypt, where 100% infection occurs in some fields, and in India, with an incidence as high as 70% and economic losses up to 51% (Johal et al. 2004). *Zea mays* (corn, maize) and *Lupinus* (lupine) are the only known hosts of *H. maydis*, although localized lesions occur on young cotton hypocotyls (Bahteem 185 cultivar) (Sabet et al. 1966). The Egyptian, Indian and Hungarian isolates of *H. maydis* differ in morphology, pathogenicity and route of infection (Warren 1983). The four clonal lineages of Egyptian isolates of *H. maydis* show diversity in amplification fragment length polymorphism (AFLP), and differ in colonization ability and virulence on maize (El-Assiuty et al. 1999; Saleh et al. 2003b; Zeller et al. 2002; Zeller et al. 2000). Three of the lineages are widely distributed throughout the country. The fourth lineage was the most virulent but least competitive on susceptible maize accessions when inoculated as a mixed inoculum of all four isolates (Zeller et al. 2002).

Late wilt disease is characterized by relatively rapid wilting of maize plants, typically at the age of 70 to 80 days, before tasseling and until shortly before maturity. First symptoms appear approximately 60 days after sowing (Sabet et al. 1970b), and include drying out of the lower parts of the plant that ascends upwards, as well as leaf yellowing and wilt, color alteration of the vascular bundles to a yellow-brown hue and then the appearance of red-brown stripes on the lower internode (Sabet et al. 1966). With disease progression, the lower stem dries out (particularly at the internodes) and has a shrunken and hollow appearance, with dark yellow to brownish macerated pith and brownish-black vascular bundles (Degani and Cernica 2014). Late wilt is often associated with infection by secondary invaders causing the stem symptoms to become more severe (El-Shafey and Claflin 1999; Samra et al. 1962). Fewer ears are produced,

and kernels that form are poorly developed (Drori et al. 2012) and may be infested with the pathogen. Seed quantity is correlated negatively to disease severity (Shehata 1976).

Spread is primarily through movement of infested soil, crop residue, or seed-borne inoculum. Spread within a field is often associated with mechanical operations such as cultivation that moves soil. *H. maydis* can persist on corn stubble for 12-15 months (Sabet et al. 1970b; Singh and Siradhana 1987b). Sclerotia are produced under low humidity and ensure long-term survival of *H. maydis* (up to 15 months) in no-till residues on the soil surface. Lupine facilitates parasitic survival of the pathogen under at least some field conditions (Botros et al. 1990; Johal et al. 2004). *H. maydis* can survive in seeds for 10 months at high temperatures and low humidity in India, but longer survival is predicted at low temperatures (Singh and Siradhana 1987a). Infested seeds can produce plants with late wilt symptoms, infest soil and result in subsequent development of late wilt in healthy seeds grown in that soil.

The most effective control of late wilt is using resistant germplasm (El-Shafey et al. 1988; Zeller et al. 2000), although some agricultural, biological and chemical controls can reduce its impact on commercial production, as detailed below. The National Maize Program at the Agricultural Research Center in Giza, Egypt identified many sources of resistance, and their release of resistant cultivars since 1980 has significantly reduced late wilt losses in Egypt (El-Shafey et al. 1988). A breeding program for resistant germ lines has existed in Israel for about a decade (Israel Northern R&D, Migal – Galilee Research Institute, Kiryat Shmona, Israel, unpublished data).

Various agricultural measures such as soil solarization, balanced soil fertility and flood fallowing can reduce disease severity and losses. Inoculum survival is restricted to the top 20 cm of soil, and survival depends primarily on the persistence in infested crop residues (Sabet et al. 1970a). Moisture management and flood fallowing may be useful cultural controls for late wilt where economically practical (Samra et al. 1966; Singh and Siradhana 1988). Balanced fertilization can reduce disease severity, although it does not provide complete control (Singh and Siradhana 1990). Many attempts were made to control the pathogen using chemical and biological methods (Abd-el-Rahim et al. 1982; Abdel-Hamid et al. 1981; El-Mehalowy et al. 2004; Sabet et al. 1972; Satyanarayana and Begum 1996; Singh and Siradhana 1989). Some fungicides that were tested worked well in pots but failed in field experiments (Abd-el-Rahim et al. 1982), while others achieved promising success (Abd-el-Rahim et al. 1982). Since *H. maydis* is a poor saprophytic competitor (Sabet et al. 1970b), various attempts at

biological control by inoculating corn seed with competitive or antagonistic organisms (*Macrophomina phaseolina*, *Trichurus spiralis*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Verticillium tricorpus*) have been evaluated (El-Assiuty et al. 1991; El-Mehalowy et al. 2004; Muhammad and Amusa 2003; Singh and Siradhana 1988); however, success on a field scale has not been demonstrated consistently. Today, there is no available effective chemical or biological method for controlling late wilt in Israel.

Species-specific PCR primers capable of distinguishing *H. maydis* from other species in the *Gaeumannomyces-Harpophora* complex have been developed previously (Saleh and Leslie 2004) and are used as a diagnostic assay of disease progress in an infested field in northern Israel (Drori et al. 2012). Here, we are conducting a controlled inspection of selected fungicides, previously shown to suppress *H. maydis in vitro* (Degani and Cernica 2014) in field plants. By assaying the disease symptoms, evaluating wilt levels, measuring yield production and using the DNA-sequence-based approach (Drori et al. 2012; Saleh and Leslie 2004), we studied the influence of these fungicides on the pathogenesis of *H. maydis* in the contaminated test field.

MATERIALS AND METHODS

Field experiments for assessing fungicide efficiency in controlling late wilt

A field experiment for assessing fungicide efficiency in controlling *H. maydis* pathogenesis in the susceptible cultivar of sweet corn Jubilee (from Pop Vriend Seeds B.V., Andijk, The Netherlands, supplied by Eden Seeds, Reut, Israel) was conducted during the spring and summer of 2009 and 2010. These two subsequent experiments were performed in the southern area (No. 10) of a maize field in Kibbutz Naot Mordechai in the Hula Valley (Upper Galilee, northern Israel), which has been known to be infested with late wilt for many years (Drori et al. 2012). Plots were arranged in the field using a randomized complete block design. Plot size differed for the years: in 2009, the area included 15 plots, each containing two rows (five plots per treatment). Each row was 60 m long and contained 6.5 maize plants m^{-1}; in 2010, the area included 50 plots (four to five plots per treatment), each containing two 40-48-m long rows (with the same plant density as in 2009). Row spacing was 96.5 cm. Seeds were pretreated with Thiram, Captan, Carboxin, Metalaxyl-M (manufactured by Rogers/Syngenta Seeds, Boise, ID, USA, supplied by CTS, Tel Aviv, Israel, quota NC7323XLF). The

field was watered in both years (unless otherwise indicated for special treatments) with 0.6 L/h using a 20 mm drip irrigation line for each row (Dripnet PC 1613F, Netafim USA, Fresno, CA). The drip points were spaced 25 cm apart. Irrigation for each treatment was controlled manually from a manifold. Four- to five-meter-long 20 mm blind affiliates equipped with a manual control valve were installed at the edge of each treatment unit near the water source and used as point of injection and mixing of the fungicides with the irrigation water. Water faucets were installed to prevent the fungicides from passing to the other treatments. The daily amount of water for each season was determined by the Penman method (Penman 1948) to 500 mm. The field was watered twice a week.

The 2009 field experiment. In the first experiment (2009), seeding was performed on April 21, 2009 and germination (with a frontal irrigation system) one day later. Plants emerged above the ground surface six days after planting. Plants were first pollinated when they reached 70% silk on June 20, 2009 (60 days after sowing). The pollination continued for one week. The field was harvested on July 14, 2009 (84 days after sowing). The Jubilee cv. field plants were treated separately with two different fungicides (Table 1): Azoxystrobin, and a mixture of 26.7% Boscalid and 6.7% Pyraclostrobin. Each treatment was done at a dosage of 150 cm^3/d and applied three times on May 7 (phenological stage V2, four visible leaves), May 21 (phenological stage V4-5, eight visible leaves, 25-30 cm plant height), and June 4 (phenological stage V8, 13-14 visible leaves, 60 cm plant height), 16, 30 and 44 days after sowing. The control group was untreated plants. Fungicides were injected directly into the drip line using a 5 ml syringe.

Wilt determination was carried out 17 (7/7/09) and 24 (14/7/09) days after fertilization (DAF) (77 and 84 days after sowing) for 100 plants in a sequence. The plants were classified as wilted when wilt symptoms appeared on the leaf whose cob is located in its axil. Yield determination was done 24 DAF (14/7/09, 84 days after sowing) and included all the upper part plant cobs in a 20-m-long section of each of the experiment rows.

The 2010 field experiment. The field experiment was repeated with modifications in the summer of the following year (2010) in the same field, and was done according to the same design as in 2009. This repetition included the susceptible maize Jubilee cv. and a partially resistant cultivar Empire from HSR (Snowy River) breeds, Queensland, Australia (supplied by Green 2000, Caesarea, Israel) used here as

an additional control. These cultivars had been tested previously for susceptibility to late wilt (Israel Northern R&D, Migal – Galilee Research Institute, Kiryat Shmona, Israel, unpublished data). Each plot contained two rows: one of the Jubilee cv. (eastern row) and one of the Empire cv. Seeding was performed on April 26, 2010 and germination was done (with a frontal irrigation system) two days later. Plants emerged above the ground surface on May 4, 2010 (eight days after planting). Empire and Jubilee cv. plants were pollinated when they reached 70% silk on June 21 and 24, 2010, respectively (59 and 62 days after sowing), and the field was harvested on July 22, 2010 (87 days after sowing).

In the 2010 field experiment, we repeated the Azoxystrobin treatment, which was found to be effective in the 2009 field experiment. The fungicide was applied as before using a drip irrigation line for each row placed adjacent to the plant rows. The Azoxystrobin treatment was done at a dosage of 150 cm^3/d and applied on May 13, May 27 and June 10, 2010 (at the same phenological stages as in the 2009 experiment) - 17, 31 and 45 days after sowing. In addition, we tested the efficiency of reduced concentrations of this fungicide and two different ways of applying the fungicide: a drip irrigation line positioned at the center of every two rows, and spraying the anti-fungal agent. We also tested the efficiency of two additional demethylation inhibitor (DMI) fungicides that were found to be effective in the agar plate assay (Degani and Cernica 2014): Flutriafol (which was also found to be effective in a seedling pathogenicity assay) and Prochloraz zinci. Concentrations were determined according to the manufacturer's recommendations, 0.75 cm^3/d and 250 cm^3/d, respectively. Both fungicides were applied three times on the same dates as the Azoxystrobin. Fungicides were injected directly into the drip line using a 5 ml syringe. The control group was untreated plants.

Alternatively the Azoxystrobin treatment was carried out at a low dosage of 50 cm^3/d applied three times, or at a high dosage of 150 cm^3/d applied only once or twice instead of the three treatments. We also applied the Azoxystrobin at a high dosage of 150 cm^3/d using a drip irrigation line positioned at the center of every two rows and spraying it in two dosages of 150 cm^3/m or 300 cm^3/m. The spraying experiment included two plots per treatment and a separate control (untreated plants) plot. Each plot contained two rows: one for the Empire cv. and one for the Jubilee cv. The spraying experiment area was located near the drip irrigation experimental area (with a safe

distance between the two experiments) and was watered with a frontal irrigation system. Overall, the 2010 experiment comprised 14 treatments:

1. Azoxystrobin 150 cm^3/d x three treatments applied on May 13, May 27 and June 10 (ABC 150).
2. Azoxystrobin 50 cm^3/d x three treatments (ABC 50).
3. Azoxystrobin 150 cm^3/d x two treatments applied on May 13 and May 27 (AB 150).
4. Azoxystrobin 150 cm^3/d x two treatments applied on May 27 and June 10 (BC 150).
5. Azoxystrobin 150 cm^3/d x one treatment applied on May 13 (A 150).
6. Azoxystrobin 150 cm^3/d x one treatment applied on May 27 (B 150).
7. Azoxystrobin 150 cm^3/d x one treatment applied on June 10 (C 150).
8. Azoxystrobin 150 cm^3/d x three treatments applied on May 27 and twice on June 10. In this treatment, the drip irrigation line was positioned at the center of every two rows (center, BCC). Due to the lower water supply (one irrigation line for two rows instead of one line per row), the plants were poorly developed (with reduced root system) when we were intend to apply the first Azoxystrobin treatment, so we decided to postpone the first fungicide treatment (planned for the 2^{nd} week) and added it twice: a single dosage during the 4^{th} week (B) and a double dosage during the 6^{th} week (CC).
9. Azoxystrobin 150 cm^3/m x one spraying treatment applied on June 10 (spraying, C 150).
10. Azoxystrobin 300 cm^3/m x one spraying treatment applied on June 10 (spraying, C 300).
11. Flutriafol 125 cm^3/d x three treatments (Flutriafol ABC).
12. Prochloraz 250 cm^3/d x three treatments (Prochloraz ABC).
13. Control – untreated plants in the drip irrigation experiment (drip irrigation NT).
14. Control – untreated plants in the spraying experiment (spraying NT).

Wilt assessment was carried out 19 days after fertilization (13/7/10) (77 days after sowing), the same way as in the 2009 field experiment. Yield assessment was carried out 28 DAF (22/7/10, 87 days after sowing) as described for the 2009 field experiment.

Molecular diagnosis of late wilt pathogenesis in the 2009 field experiment

Three plants were collected arbitrarily from the Jubilee cv. plants at approximately three-week intervals from day 20 after sowing onwards. The last two samplings were made at two-week intervals in order to inspect disease eruption more closely. Sampling was made on days 20 (11/5/09), 40 (31/5/09), 61 (21/6/09), 75 (5/7/09) and 89 (19/7/09) after seeding. Different plant tissues (root, stem, leaf and seed) were sterilized separately with 70% ethanol and then washed with autoclaved DDW. The plants were ground separately in a mortar with pestle, and DNA was extracted and analyzed in three independent replications, as described below.

DNA was obtained with the Extract-N-amp plant PCR kit (Sigma, Rehovot, Israel) according to the manufacturer's instructions. For positive controls, we used 7-day-old fungal cultures grown on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) at 28^{o}C in complete darkness for six days. PCR was performed to amplify a specific *H. maydis* segment (Drori et al. 2012; Saleh and Leslie 2004) with a Rapidcycler (Idaho Technology, Salt Lake City, UT, USA). We modified an existing molecular assay (Saleh and Leslie 2004) by choosing a new set of primers (A200a and Am42/43) using a different reaction mixture and altering the cycling condition as described below (adapted from Degani and Cernica 2014). This new sequence is a major part of a larger AFLP fragment that was proven earlier to be species-specific (Drori et al. 2012; Saleh et al. 2003b; Zeller et al. 2000). The A200a primer set – [A200a-for (forward primer): 5'-CCTAGTAGTCCCGACTGTTAGG-3', A200a-rev (reverse primer): 5'-TTGGTTCACCGTCTTTTGTAGG-3'] amplifies a specific (Saleh and Leslie 2004) *H. maydis* segment. The Am42/43 primer set [Am42 (anti-sense primer): 5'-CAACTACGAGCTTTTTAACTGC-3', Am43 (sense primer): 5'-CAAATTACCCAATCCCGACAC-3'] amplifies eukaryotic ribosomal DNA (18S rRNA gene product, rDNA, Fromont-Racine et al. 2003) and was used for positive control. Reaction mixtures were contained in a total volume of 50 µl: 1 µl of each primer (20 µM of each primer), 25 µl Red Load Taq Master (Larova, Teltow, Germany), 3 µl DNA sample and 20 µl DDW. Cycling conditions for all primer pairs were 94^{o}C for 2 min, followed by 35 cycles of 94^{o}C for 30 sec, 55^{o}C for 30 sec, 72^{o}C for 1 min, and a final step of 72^{o}C for 5 min. After PCR, a specific 200-bp amplified DNA band and a ribosomal DNA (18s rDNA, used as positive control) were identified by electrophoresis on an Agarose gel.

RESULTS

Field experiment for assessing fungicide efficiency in controlling late wilt

To examine the effect of different fungicides on disease progression in a contaminated field, we conducted a field experiment on the Kibbutz Naot Mordechai maize field in the spring and summer of 2009 and 2010. The first experiment (2009) included only the susceptible Jubilee cv., while the 2010 sampling program included two representative maize cultivars: Empire cv., a relatively late wilt-resistant cultivar, and the Jubilee cv. Early signs of the disease began to appear in the susceptible Jubilee cv. approximately two months after sowing, as discussed in more detail below.

The 2009 field experiment. Usually the more severe symptoms of wilt occur adjacent to the flowering and first stages of seed development. In the 2009 field experiment, the evident drying out signs of the lower part of the stem (brown strips) appeared in the untreated susceptible Jubilee cv. about 10 days later than expected (according to our experience in commercial fields in northern Israel), one week after flowering (67 days after sowing). The first symptoms evident on the leaves of this cultivar appeared 3-5 days later (Fig. 1). At that time, the plants treated with a mixture of 26.7% Boscalid and 6.7% Pyraclostrobin exhibited only slight symptoms and those treated with the systemic fungicide Azoxystrobin were healthy (showed no sign of disease, Fig. 1). The percentage of wilting assessed 17 and 24 DAF (77 and 84 days after sowing, respectively) and yield assessment done 24 DAF give a more accurate estimation of these observations. In the control treatment, the percentage of wilting plants was 32% and 94%, respectively, and cob yield was 995 kg/d (Fig. 2). At 17 DAF, a significant ($p=0.02$) decrease in wilting percentage was measured in the 26.7% Boscalid and 6.7% Pyraclostrobin mixture treatment (16% wilting), but 24 DAF, the difference between the control and this treatment disappeared, and the wilting percentage of this treatment reached 94% (Fig. 2). Crop yield assessment of the plots treated with this fungicide mixture showed no remarkable change compared to the control (1018 kg/d, Fig. 2). On the other hand, the Azoxystrobin treatment was highly effective compared to the control ($p=0.002$ 17 DAF and $p=1.1*10^{-5}$ 24 DAF) and resulted in almost no signs of wilting (1.4% of wilting 17 DAF, and 14% of wilting 24 DAF, Figs. 1, 2). Even more remarkable was an increase in crop yield 24 DAF, which reached 100% more than the control (1966 kg/d, $p=1.8*10^{-5}$, Fig. 2). This Jubilee cv.

maize crop yield reached conventional yield measured in the past in healthy fields in the area (Drori et al. 2012).

Despite the success of the Azoxystrobin treatment in preventing late wilt disease symptoms, the molecular diagnosis carried out to detect pathogen DNA in the host tissues clearly identified fungus in the root and stem of the plants from day 75 onwards in both the control and the Azoxystrobin treatments (Fig. 3). The amount of DNA measured was slightly higher in the root compared to the stem, but was identical in both the control and the fungicide treatments (Fig. 3). The same DNA measurement results were obtained for the in the 26.7% Boscalid and 6.7% Pyraclostrobin mixture treatment (data not shown).

The 2010 field experiment. The field experiment was repeated in the following summer in order to establish the previous Azoxystrobin treatment results, modifying the application method and inspecting two more fungicides that were not included in the first experiment for practical reasons. Similar to the 2009 field experiment, the first symptoms appeared later than the expected date of appearance in commercial fields. In the untreated control group, the first late wilt symptoms, mainly coloring alternation in the xylem tissue of the first internode, were noticed one week after fertilization (69 days after sowing) (data not shown). Evident withering symptoms in the leafage (especially in the lower parts) were observed 15-17 DAF (77-79 days after sowing). Wilting assessment at 19 DAF (77 days after sowing) revealed that the triple Azoxystrobin treatment caused an over 85% decrease in wilting percentage of this plant (Fig. 4B) compared to the control group. The two other fungicide treatments that were introduced and evaluated in this experiment, Flutriafol and Prochloraz, did not result in significantly different wilting from the control group (Fig. 4B). A similar picture was revealed upon examining crop production. The Azoxystrobin treatment caused an increase of 63% in crop yield compared to the conventional level in healthy fields (Drori et al. 2012) (1.942 kg/d, Fig. 4A) and was similar to the level achieved in the 2009 field experiment (Fig. 2A). The two other fungicide treatments, Flutriafol and Prochloraz, resulted in a crop yield similar to the 1222 kg/d yield of the control treatment (Fig. 4A).

Modifications to the Azoxystrobin treatment were carried out in order to decrease costs and make it more profitable for the grower. Nevertheless, the application of antifungal agent once (150A, 150B, 150C, Fig. 5) or twice (150AB, 150BC, Fig. 5) instead of three times (150ABC, Fig. 5) was still effective in reducing wilting symptoms

and increasing yield compared to the control group, but none were equal to the effectiveness of the triple full dosage Azoxystrobin treatment (Fig. 5). Interestingly, the addition of a dosage of 150 cm^3/d of antifungal agent once instead of three times was more effective in the 4^{th} or 6^{th} weeks (150 B and 150C, respectively, Fig. 5) than in the 2^{nd} week (150A, Fig 5). Although a single application of Azoxystrobin two weeks after sowing (150A) is completely ineffective with low yields as the control (Fig. 5), its application is significant for late wilt disease prevention as part of a three-treatment set since the dual dilated treatment (150 BC) didn't match the effectiveness of the triangular treatment (150 ABC, Fig. 5).

Decreasing the Azoxystrobin dosage to one third (50 cm^2/d, 50ABC, Fig. 5) or applying the fungicide by positioning the drip irrigation line at the center of every two rows (150 Center, Fig. 5) reduced wilting symptoms to the level of a single or double treatment but still did not result in improved yield (Fig. 5). Appling Azoxystrobin by spraying was only effective at the high dosage of 300 cm^3/d, although the apparently significant reduction in disease symptoms using this treatment achieved only an 18% increase in yield (Table 2). The relatively resistant maize cultivar, Empire cv., used in the spraying experiment as a control group showed no signs of wilting but unexpectedly had a 32% increase in yield at the low 150 cm^3/d dosage spraying treatment.

DISCUSSION

We have been inspecting maize plants that developed in infested grounds over the past five years (growing seasons). First emergence and progression of disease symptoms are subject to environmental and host physiological changes. In susceptible maize cultivars such as Jubilee, the first symptoms of wilting usually appeared in the field 50-60 days after sowing (Israel Northern R&D, Migal – Galilee Research Institute, Kiryat Shmona, Israel, unpublished data), shortly before the tasseling stage, as reported earlier in Egypt (Sabet et al. 1970b). Nevertheless, when we used a drip irrigation line (the 2009 and 2010 field experiments reported here) instead of a frontal irrigation system, plants reached 70% silk 60 days after sowing and the first disease symptoms appeared only 10 days later (70 days after seeding). This indicates a possible influence of irrigation on disease outburst. Indeed, in the 2010 field experiment, untreated plots watered with a frontal irrigation system (the spraying experiment, Table 2) showed 44% wilt and 0.57 t/d yield, while the untreated plots watered with a drip line showed a slight decrease in

wilt (41%) but a significant increase in yield (1.22 t/d). Reviewing the literature supports the conclusion that low water potential is one of the most important factors enhancing disease progression. Flood fallowing increases anaerobic conditions, stimulates lytic organisms to degrade sclerotia and reduces survival potential. Early sowing of corn in Egypt reduced late wilt (El-Shafey et al. 1988), while late summer planting reduced disease severity in India (Singh and Siradhana 1988). Unfavorable soil conditions with low rainfall may be the determining factor with a reported date of seeding effects (Singh and Siradhana 1988). Moisture stress is a major predisposing factor for late wilt (Abd El-Rahim et al. 1998), and frequent watering or saturated soils reduced late wilt (Samra et al. 1966). Corn did not develop late wilt following paddy-cultivated rice, which increases the availability of Mn for subsequent crops, although *H. maydis* is also sensitive to low oxygen conditions (Samra et al., 1966).

In the past, many attempts have been made to eradicate the pathogen in chemical and biological ways (Abd-el-Rahim et al. 1982; Abdel-Hamid et al. 1981; El-Mehalowy et al. 2004; Sabet et al. 1972; Satyanarayana and Begum 1996; Singh and Siradhana 1989). Some fungicides tested worked well in pots but failed in field experiments (Abd-el-Rahim et al. 1982). At present, the most economically effective management of late wilt is through the development of genetically resistant corn lines (El-Shafey et al. 1988). However, little information is available in the literature on the pathogen's development in those apparently asymptomatic maize cultivars. Here, we used *H. maydis*-unique nucleotide sequence, which proved earlier to be species-specific (Saleh et al. 2003a), as a diagnostic tool to track pathogen spread in the host tissues under the influence of selected fungicides (shown for the Azoxystrobin treatments in Fig. 3). As previously described (Drori et al. 2012), the pathogen spreads in moderately resistant Royalty cv. plant tissues, root stalk, leaves and stalks in a similar way to the pathogenesis in the sensitive maize Jubilee cv., but the relatively lesser amounts of pathogen DNA in Royalty cv. imply that it does not become well established. Currently it is unclear how the host has restricted the ability of the pathogen to grow and induce disease symptoms. Similarly, the Azoxystrobin fungicide treatment (Figs. 1-3) applied in the 2009 growth season prevented disease symptoms but didn't abolish fungal establishment in the host tissues (Fig. 3).

Since *H. maydis* is capable of undergoing pathogenic variations (El-Assiuty et al. 1988; Zeller et al. 2002), new virulent strains could develop, so seeking alternative ways to control the disease is an ongoing process. To exemplify this importance, a

virulent lineage exists in Egypt that is a threat to some resistant maize cultivars (Zeller et al. 2002), while in Israel the relatively resistant maize cultivar, Royalty, which has become the leading maize cultivar since the late disease outburst, started to show wilting symptoms in the summer of 2010 and caused significant economic damage when a disease outburst caused its collapse in Beit She'an (Jezreel Valley, Lower Galilee region in Israel) maize fields in the summer of 2013 (Israel Northern R&D, Migal – Galilee Research Institute, Kiryat Shmona, Israel, unpublished data). Therefore, the need to identify new effective compounds against the late wilt causing agent is urgent and requires continuous efforts. The presence of the pathogen in the host tissues of the successfully treated plants (Figs. 1-3) is hinting at the risk that the pathogen will develop immunity to the fungicides. Moreover, combining two or more fungicides with a different mode of action may be crucial for preventing the development of fungal resistance.

One example is Azoxystrobin, a member of the class of Qo-inhibiting fungicides (QoIs), which is probably the most successful class of agricultural fungicides (Fernández-Ortuño et al. 2010). Unfortunately, the rapid development of resistance to these fungicides and consequent control failure has become increasingly problematic (see, for example, in *Magnaporthe grisea* (Avila-Adame and Koller 2003)).

Further research with this and other fungicides is important for controlling the disease. These fungicide assays could include potted plant assays (as demonstrated in Degani and Cernica 2014) and field assays in which fungicides may be applied using the irrigation system, as demonstrated in this work. Systemic fungicides and their fungitoxic products are translocated to corn leaves within two days and can persist in corn roots for 90 days; however, field results have been generally disappointing unless the fungicide is applied several times during the growing season (Singh and Siradhana 1989). The cost and labor required for frequent fungicide applications to corn make chemical control prohibitively expensive. Indeed, the total amount of Azoxystrobin used in the 2009 field experiments (112.5 g/d) as well as the use of a drip irrigation line for each row are not economically realistically, and the control method demonstrated should be modified and improved before it could be feasible.

Attempts at achieving this goal were made in the following year (2010). A series of experiments showed that success in controlling maize wilting disease requires high doses and series applications of the systemic fungicide Azoxystrobin as supplementation to the drip irrigation water provided adjacent to the row. Any change

in one of these parameters adversely affects treatment efficiency. Moreover, failure of three other fungicides tested here (Figs. 2, 4) left us with a single-molecule based fungicide (Azoxystrobin) to restrain the disease.

Future efforts should focus on locating additional effective compounds that could be applied in field conditions and that could be integrated into pesticides with Azoxystrobin. Pest control that combines chemical groups with different mechanisms of action is essential for delaying pathogen resistance and for the development of lower dose preparations (due to synergism).

ACKNOWLEDGMENTS

We would like to thank Dr. Onn Rabinovitz (Israel Ministry of Agriculture and Rural Development, Consultation Service) for his helpful advice and Asaf Blatman (Tel-Hai College, Israel) for his technical assistance. This work was supported by a research grant from the Israel Plant Council, Ministry of Agriculture and a research grant from the Jewish National Fund (Keren Kayemeth LeIsrael). The experiments comply with the current laws of Israel.

REFERENCES

Abd-el-Rahim M. F., Sabet K. A., El-Shafey H. A., El-Assiuty E. M. (1982). Chemical control of the late-wilt disease of maize caused by *Cephalosporium maydis*. Agric Res Rev, 60, 31-49

Abd El-Rahim M. F., Fahmy G. M., Fahmy Z. M. (1998). Alterations in transpiration and stem vascular tissues of two maize cultivars under conditions of water stress and late wilt disease. Plant Pathology, 47, 216-223

Abdel-Hamid M. S., Abdel-Momeim M. F., El-Shafey H. A., El-Deeb S. T. (1981). Biological control of late-wilt disease of maize caused by *Cephalosporium maydis*. Agric Res Rev, 59, 253-260

Avila-Adame C., Koller W. (2003). Characterization of spontaneous mutants of *Magnaporthe grisea* expressing stable resistance to the Qo-inhibiting fungicide azoxystrobin. Current Genetics, 42, 332-338

Botros S. E. D., Mohamed M. S., El-Shabrawy A. M., Abd-Elrakik A. (1990). Effect of intercropping maize with certain legumes on the incidence of maize root and stalk rots. Assiut J Agric Sci, 21, 155-170

Degani O., Cernica G. (2014). Diagnosis and control of *Harpophora maydis*, the Cause of late wilt in maize. Advances in Microbiology, 4, 94-105

Drori R., Sharon A., Goldberg D., Rabinovitz O., Levy M., Degani O. (2012). Molecular diagnosis for *Harpophora maydis*, the cause of maize late wilt in Israel. Phytopathologia Mediterranea, 52, 16-29

El-Assiuty E. M., El-Hamahmy A. A., El-Sharkawy A. Y. (1991). *Bacillus subtilis*, *Pseudomonas fluorescens* and *Verticillium tricorpus* as biological agents against late-wilt of maize. Egypt J Appl Sci, 6, 8245-8829

El-Assiuty E. M., El-Shafey H. A., Ismael A.-S., Fahmy Z. M. (1988). Pathogenic variation in *Cephalosporium maydis*. Phytopathology, 88, S25

El-Assiuty E. M., Ismael A. M., Zeller K. A., Leslie J. F. (1999). Relative colonization ability of greenhouse-grown maize by four lineages of *Cephalosporium maydis* from Egypt. Phytopathology, 89, S23

El-Mehalowy A. A., Hassanein N. M., Khater H. M., Daram El-Din E. A., Youssef Y. A. (2004). Influence of maize root colonization by rhizosphere actinomycetes and yeast fungi on plant growth and on the biological control of late wilt disease. Inter J Agric Biol, 6, 599-605

El-Shafey H. A., Claflin L. E. (eds) (1999) Late Wilt. APS Press, St. Paul, Mn

El-Shafey H. A., El-Shorbagy F. A., Khalil I. I., El-Assiuty E. M. (1988). Additional sources of resistance to the late-wilt disease of maize caused by *Cephalosporium maydis*. Agric Res Rev, Egypt, 66, 221-230

Fernández-Ortuño D., Torés J. A., De Vicente A., Pérez-García A. (2010). Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. International Microbiology, 11, 1-9

Fromont-Racine M., Senger B., Saveanu C., Fasiolo F. (2003). Ribosome assembly in eukaryotes. Gene, 313, 17-42

Gams W. (1971) Cephalosporium-artige Schimmelpilze (Hyphomycetes). G. Fischer, Stuttgart

Johal L., Huber D. M., Martyn R. (2004) Late wilt of corn (maize) pathway analysis: intentional introduction of *Cephalosporium maydis*. In: Pathways Analysis for the Introduction to the U.S. of Plant Pathogens of Economic Importance.

Michail S. H., Abou-Elseoud M. S., Nour Eldin M. S. (1999). Seed health testing of corn for *Cephalosporium maydis*. Acta Phytopathologica et Entomologica Hungarica, 34, 35-42

Molinero-Ruiz M. L., Melero-Vara J. M., Mateos A. (2011). *Cephalosporium maydis*, the cause of late wilt in maize, a pathogen new to Portugal and Spain. Plant Dis, 94, 379-379

Muhammad S., Amusa N. A. (2003). *In vitro* inhibition of growth of some seedling blight inducing pathogens by compost-inhabiting microbes. Afr J Biotechnol, 2 161-164

Payak M. M., Lal S., Lilaramani J., Renfro B. L. (1970). *Cephalosporium maydis* - a new threat to maize in India. Indian Phytopathol, 23, 562-569

Pecsi S., Nemeth L. (1998). Appearance of *Cephalosporium maydis Samra Sabet and Hingorani* in Hungary. Mededelingen Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Universiteit Gent, 63, 873-877

Penman H. L. (1948). Natural evaporation from open water, bare soil and grass. Proceedings of the Royal Society of London Series A Mathematical and Physical Sciences, 193, 120-145

Sabet K. A., Samra A. S., Abdel-Rahim M. F. (1972). Systemic action of benomyl against late-wilt disease of maize. Ann Appl Biol, 71, 211-218

Sabet K. A., Samra A. S., Dawood N. A. (1966) Combined infection with stalk-rot fungi. In: Samra A. S., Sabet K. A. (eds) Investigations on stalk-rot disease of maize in UAR. Ministry of Agriculture, Government Printing Offices, Cairo, pp 195-204

Sabet K. A., Samra A. S., Hingorani M. K., Mansour I. M. (1961). Stalk and root rots of maize in the United Arab Republic. FAO Plant Prot Bull, 9, 121-125

Sabet K. A., Samra A. S., Mansour I. M. (1970a). Saprophytic behaviour of *Cephalosporium maydis* and *C. acremonium*. Annals of Applied Biology, 66, 265-271

Sabet K. A., Zaher A. M., Samra A. S., Mansour I. M. (1970b). Pathogenic behaviour of *Cephalosporium maydis* and *C. acremonium*. Ann Appl Biol, 66, 257-263

Saleh A. A., Leslie J. F. (2004). *Cephalosporium maydis* is a distinct species in the Gaeumannomyces-Harpophora species complex. Mycologia, 96, 1294-1305

Saleh A. A., Zeller K. A., Ismael A.-S. M., Fahmy Z. M., El-Assiuty E. M., Leslie J. F. (2003a). Amplified fragment length polymorphism diversity in *Cephalosporium maydis* from Egypt. Phytopathology, 93, 853-859

Saleh A. A., Zeller K. A., Ismael A. S., Fahmy Z. M., El-Assiuty E. M., Leslie J. F. (2003b). Amplified fragment length polymorphism diversity in *Cephalosporium maydis* from Egypt. Phytopathology, 93, 853-859

Samra A. S., Sabet K. A., Abdel-Rahim M. F. (1966) Effect of soil conditions and cultural practices on infection with stalk rots. U.A.R. Ministry of Agric. Government Printing Offices, Cairo, Egypt

Samra A. S., Sabet K. A., Hingorani M. K. (1962). A new wilt disease of maize in Egypt. Plant Dis Rep, 46, 481-483

Satyanarayana E., Begum H. (1996). Relative efficacy of fungicides (seed dressers) and irrigation schedule for the control of late wilt of maize. Curr Res Univ Agric Sci Bangalore, 25, 59-60

Shehata F. A. (1976) The inheritance of resistence to late wilt caused by *Cephalosporium maydis* in some corn lines Fac Agric. Al-Azhar, Cairo

Singh S. D., Siradhana B. S. (1987a). Influence of some environmental conditions on the development of late wilt of maize induced by *Cephalosporium maydis*. Indian J Mycol Pl Pathol, 17, 1-5

Singh S. D., Siradhana B. S. (1987b). Survival of *Cephalosporium maydis*, incitant of late wilt of maize. Indian J Mycol Pl Pathol, 17, 83-85

Singh S. D., Siradhana B. S. (1988). Date of sowing in relation to late wilt disease of maize. Indian Phytopathol, 41, 489-491

Singh S. D., Siradhana B. S. (1989). Chemical control of late wilt of maize induced by *Cephalosporium maydis*. Indian J Mycology Pl Path, 19, 121-122

Singh S. D., Siradhana B. S. (1990). Effect of macro and micronutrients on the development of late wilt of maize induced by *Cephalosporium maydis*. Summa Phytopath, 16, 140-145

Warren H. L. (1983). Potential disease problems: late wilt of maize. Phytopathology, 73, 782

Zeller K. A., Abou-Serie M. I., El-Assuity E. M., Fahmy Z. M., Bekheet F. M., Leslie J. F. (2002). Relative competitiveness and virulence of four clonal lineages of *Cephalosporium maydis* from Egypt toward greenhouse-grown maize. Plant Dis, 86, 373-378

Zeller K. A., Jurgenson J. E., El-Assiuty E. M., Leslie J. F. (2000). Isozyme and - amplified fragment length polymorphisms from *Cephalosporium maydis* in Egypt Phytoparasitica, 28, 121-130

Table 1: Fungicides used in this study*

Fungicide	Manufacturer, Supplier	Active Ingredient (common name) and CAS number	Group Name	Chemical Group	Target Site of Action	Active Ingredient Concentration (g/l)	Inspected in the Field
Amistar®	Syngenta (Basel, Switzerland), Makhteshim Agan (Airport City, Israel)	Azoxystrobin (CAS no. 131860-33-8)	QoI-fungicides (quinone outside inhibitors)	Methoxy-acrylates	Complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (*cyt b gene*) (inhibition of mitochondrial respiration)	250	2009: 150 cm^3/d x3 2010: 50 cm^3/d x3 150 cm^3/d x1, x2, x3
Signum W.G.®	BASF (Ludwigshafen, Germany), Agan (Ashdod, Israel)	26.7% Boscalid (CAS no. 188425-85-6) + 6.7% Pyraclostrobin (CAS No. 175013-18-0)	SDHI (Succinate dehydrogenase inhibitors) QoI-fungicides (Quinone outside Inhibitors)	Pyridine-carboxamides Methoxy-carbamates	Complex II: succinate-dehydro-genase Complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (*cyt b gene*)	267 67	2009: 150 g/d x3
Hosen	Cheminova (Lemvig, Denmark), Makhteshim Agan (Airport City, Israel)	Flutriafol (CAS no. 87676-93-5)	DMI-fungicides (demethylation inhibitors)	Triazoles	Disrupt C14-demethylation in sterol biosynthesis (*erg11/cyp51*)	125	2010: 75 cm^3/d x3
Sportec / Mirage 45 ECNA	Merhav Agro Ltd. (Herzliah Israel) / Makhteshim Agan (Airport City, Israel)	Prochloraz/Prochloraz zinci (CAS no. 68444-81-5)	DMI-fungicides (demethylation inhibitors)	Imidazoles	C14-demethylation in sterol biosynthesis (*erg11/cyp51*)	450	2010: 250 cm^3/d x3

* This information is based on the fungicides data sheet published by the manufacturer and the Fungicide Resistance Action Committee (FRAC) Code List.

Table 2: **The efficiency of the Azoxystrobin spraying mode in controlling late wilt of maize (2010 field experiment)** [a]

Azoxystrobin (cm^3/d)	Yield (ton/d)		Wilting Plants (%)	
	Empire cv.	Jubilee cv.	Empire cv.	Jubilee cv.
NT [b]	2.24	0.577	0	44
150	2.951	0.583	0	47
300	2.219	0.686	0	12

[a] Dehydration assessment was done 19 and 22 days after fertilization in Jubilee cv. and Empire cv, respectively. Yield assessment was done 28 days after fertilization. Results represent the average of two replications.

[b] NT – control – untreated plots.

FIGURES

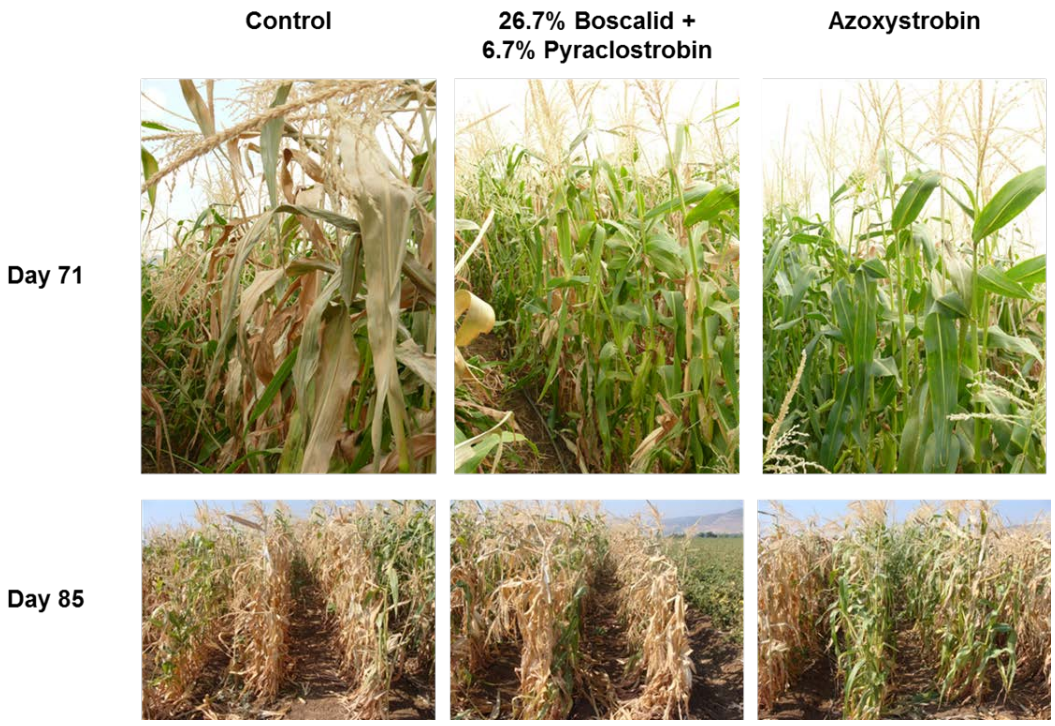

Fig. 1. The 2009 field experiments for assessing fungicide efficiency in controlling late wilt. The experiment was conducted in an infested sweet corn field in the Hula Valley (Upper Galilee, northern Israel). The Jubilee cv. field plants were treated separately with two different fungicides (Table 1), Azoxystrobin and a mixture of 26.7% Boscalid and 6.7% Pyraclostrobin. Each treatment was done at a dosage of 150 cm^3/d and applied three times. The control (untreated) and fungicide treated groups of Jubilee maize plants in the field were photographed 71 and 85 days after sowing.

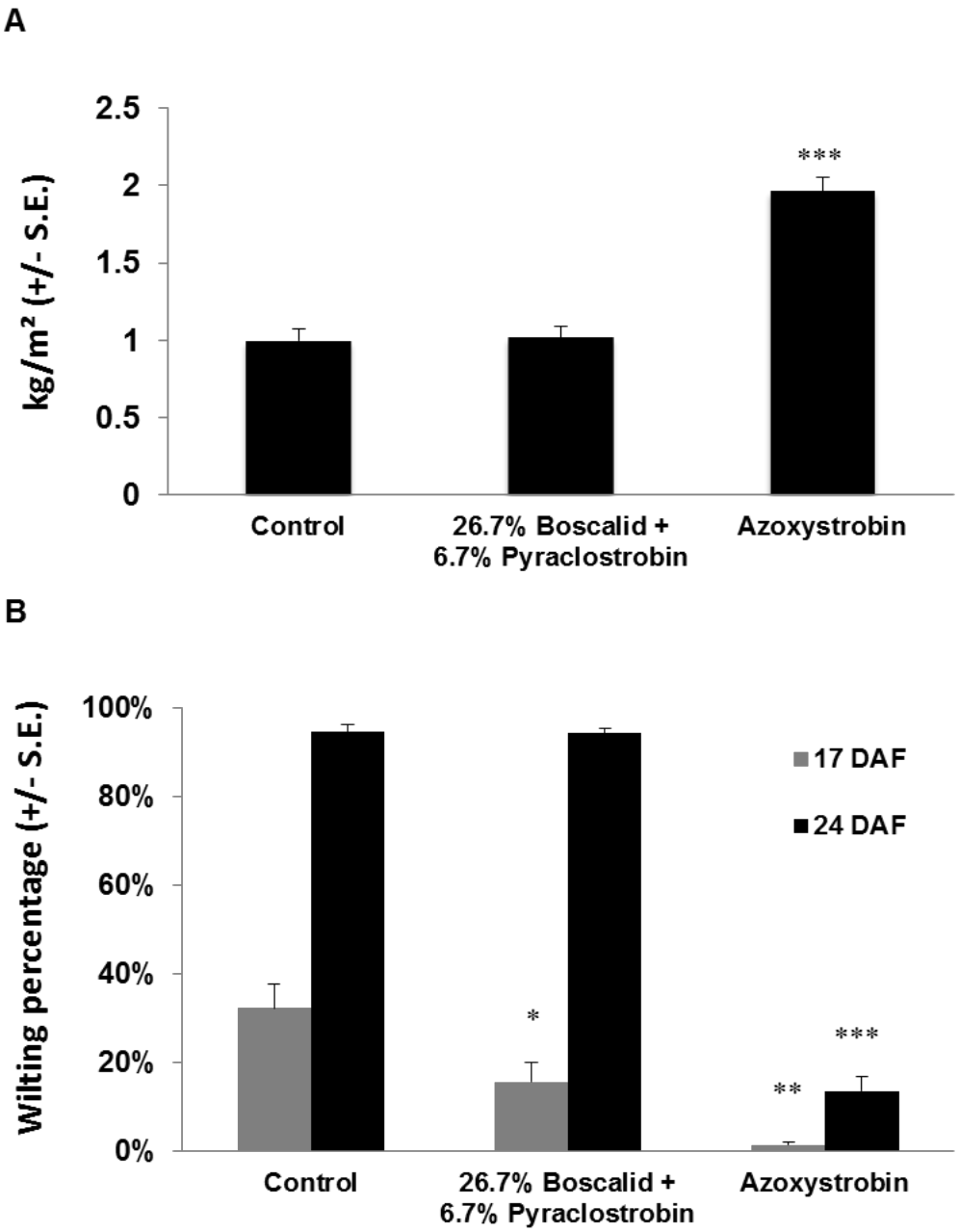

Fig. 2. Yield and wilt assessments of the 2009 field experiments. **A**. Yield assessment (in kg/m^2) done 24 days after fertilization (DAF) (14/7/09, 84 days after sowing) that includes all upper part plant cobs in a 20-m-long section of each of the experiment rows. **B**. Wilt assessment done 17 (7/7/09) and 24 (14/7/09) DAF (77 and 84 days after sowing) for 100 plants in a sequence. The plants were classified as wilted when wilt symptoms appeared on the leaf whose cob is located in its lap. Asterisks represent significant (*P=0.02, ** P=0.002, *** P<2∗10^{-5}) differences from the control. Bars indicate standard error.

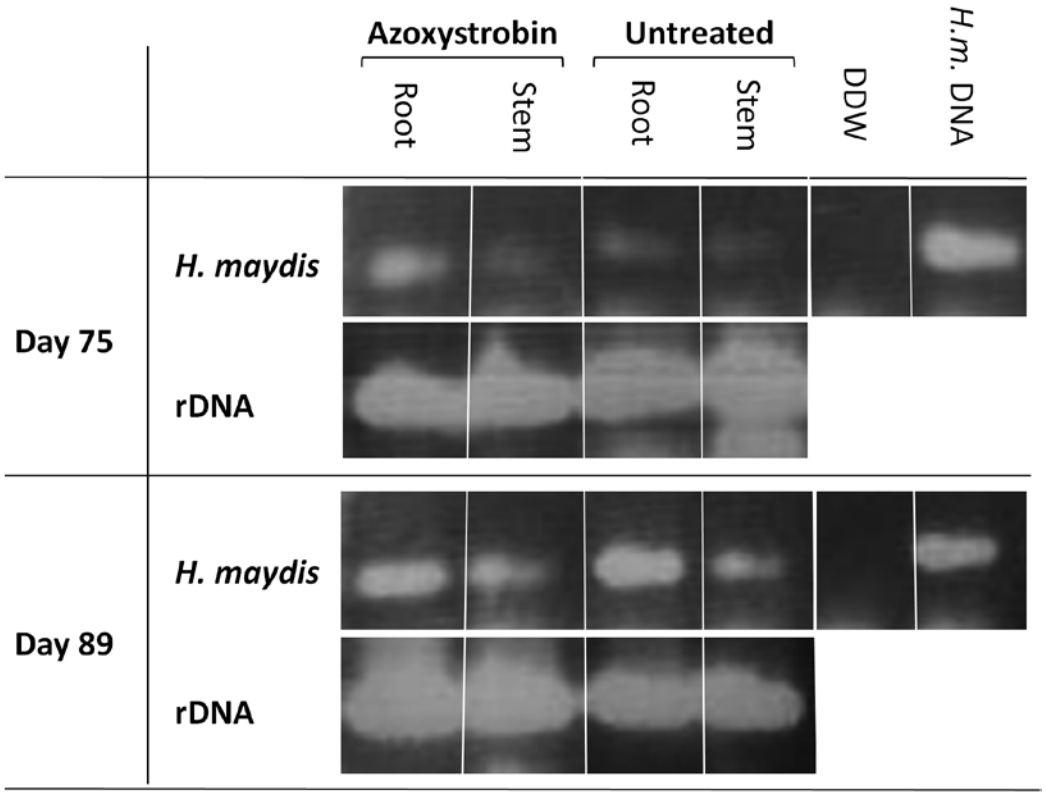

Fig. 3. Molecular diagnosis of late wilt in maize plant samples in the 2009 field experiments. Root and stem samples were inspected for the presence of the pathogen using PCR amplification of the unique *H. maydis* oligonucleotide (marked as *H. maydis*) 75 (upper panel) and 89 (lower panel) days after sowing. rDNA – amplified 18S eukaryotic ribosomal DNA; DDW – double-distilled water used as a template in the PCR mixture to ensure the absence of DNA contamination; and *H. m.* DNA – DNA from 7-day-old *H. maydis in vitro* growth cultures used here as positive control for the unique DNA amplification.

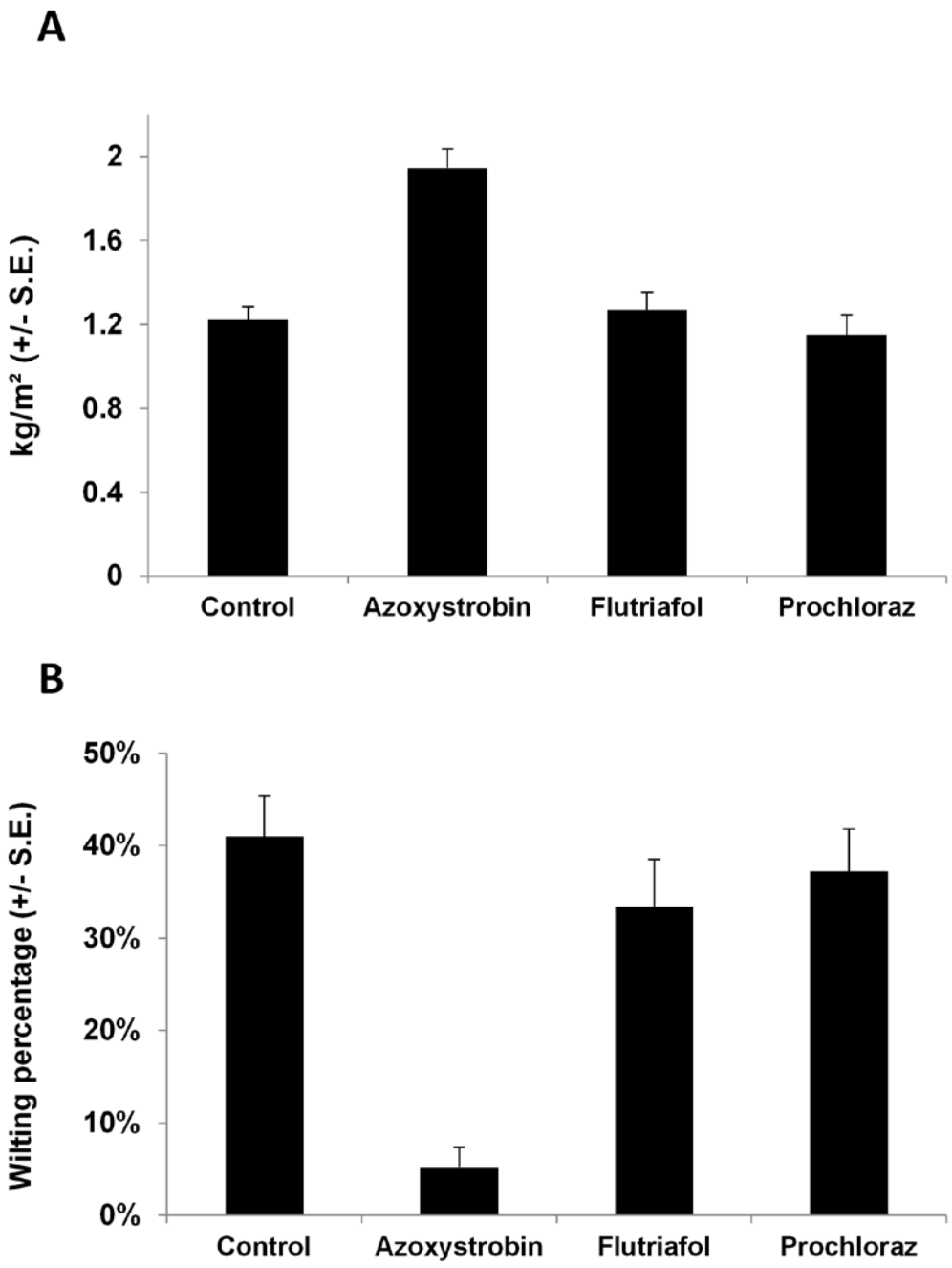

Fig. 4. Yield and wilt assessments of the 2010 field experiments. In addition to Azoxystrobin (also shown in Fig. 4), the experiments included two additional demethylation inhibitor (DMI) fungicides that were found to be effective in agar plate assay: Flutriafol and Prochloraz zinci. Yield assessment (**A**) done 28 DAF (22/7/10, 87 days after sowing) and wilt assessment (**B**) done 19 DAF (13/7/10, 77 days after sowing), as described for the 2009 field experiment (Fig. 1).

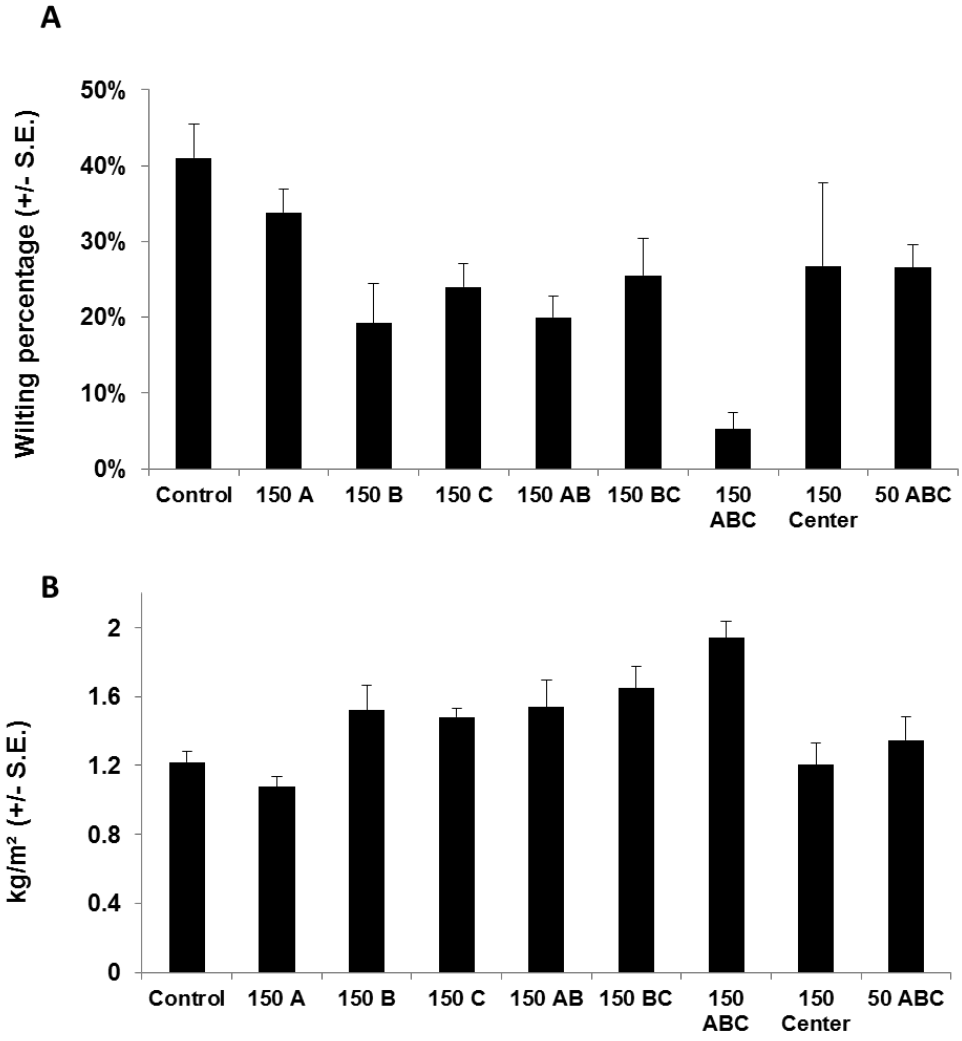

Fig. 5. Assessment of Azoxystrobin in reduced concentrations and alternative application methods in the 2010 field experiments. This experiment was part of the large 2010 field experiment, illustrated in Figure 4, and included nine treatments: the full dosages of Azoxystrobin 150 cm^3/d x three treatments (ABC 150), done in the successful 2009 field experiment (Fig. 2); a third dosage of Azoxystrobin 50 cm^3/d x three treatments (ABC 50); Azoxystrobin 150 cm^3/d x two treatments applied twice in the 2^{nd} and 4^{th} week from seeding (AB 150) or the 4^{th} and 6^{th} week from seeding (BC 150); Azoxystrobin 150 cm^3/d x one treatment applied once in the 2^{nd} (A 150), 4^{th} (B 150) or 6^{th} (C 150) week from seeding; Azoxystrobin 150 cm^3/d x three treatments applied in the 4^{th} week from seeding and twice in the 6^{th} week from seeding where the drip irrigation line was positioned at the center of every two rows (C 150); and control – untreated plants in the drip irrigation experiment. **A**. Wilt assessment done 19 DAF (13/7/10, 77 days after sowing) in the same way as the 2009 field experiment. **B**. Yield

assessment done 28 DAF (22/7/10, 87 days after sowing) as described for the 2009 field experiment.