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Stemphylium sp., the cause of yellow leaf spot disease in sugar beet (*Beta vulgaris* L.) in the Netherlands

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Abstract During the summer of 2007, a distinctive type of yellow leaf spot was observed for the first time in sugar beet (*Beta vulgaris* L.) in the Netherlands. In the following years a rapid spread over all regions of the Netherlands was reported. The infestation appears in June-August on the leaves of sugar beet and starts with small, irregular, yellow spots. Subsequently the yellow spots become necrotic from the centre of the lesion outward, with the tissue becoming brown (or brownish). The spots spread over the leaves and infest all the leaves of the plant. Due to the loss of leaves the size of the canopy declines and yield loss occurs. Research was conducted to identify the causal agent. Climate room trials with isolates obtained from infested sugar beet leaves showed that *Stemphylium* sp. was able to infest leaves of healthy growing sugar beet plants with brown spots, similar to the necrotic spots seen in the field. From the leaf spots in the climate room trials, *Stemphylium* sp. was re-isolated, according to Koch's postulates. Thus, it was confirmed that this *Stemphylium* sp. is a primary pathogen for sugar beet. In field trials of fungicide efficacy a sugar yield loss of up to 42 % (a financial yield loss of 51 %) was found. Field trials show that it is hard to control this *Stemphylium* sp. with the fungicides registered for sugar beet in the Netherlands and efficacy of control of *Stemphylium* sp. differs between fungicides with active ingredients belonging to the same chemical class. In addition to sugar beet, potato (*Solanum tuberosum* L.), white mustard (*Sinapsis alba* L.), red beet (*Beta vulgaris* L.), spinach (*Spinacia oleracea* L.) and fat hen (*Chenopodium album* L.) were identified as hosts in an assay of plants grown and inoculated in climate rooms.

Keywords Stemphylium · Sugar beet · Yield loss · Fungicide efficacy · Emerging pathogens

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Introduction

Plant pathogens can have a devastating effect on crop yield. Even when crop protection measures are applied, yield loss worldwide is estimated at 34 % due to pests and diseases (Oerke and Dehne 2004). Yield losses in Dutch sugar beet (*Beta vulgaris* L.) production due to plant pathogens and pests, were found to be 24 %, although crop protection measures were taken, stressing the importance of growers' management (Hanse et al. 2011).

Yield losses that occur in sugar beet despite crop protection measures, can be due to management decisions (Hanse et al. 2011; Oerke and Dehne 2004), selection of resistance-breaking strains of the pathogen (Koenig et al. 2009; Pferdmenges et al. 2009; Liu et al. 2005; Bornemann et al. 2014), development of resistance to pesticides by the pathogen and pests (Georgopoulos and Dovas 1973; Ruppel and Scott 1974; Giannopolitis 1978; Sawicki et al. 1978; Bugbee 1995, 1996; Devonshire et al. 1998; Secor et al. 2010;

Bolton et al. 2013), a changing climate (Pautasso et al. 2012) and the emergence (or introduction) of new pathogens (Anderson et al. 2004). The aim of this paper is to describe the emergence of the yellow leaf spot disease in sugar beet production in the Netherlands, research on the identification of the causal agent and the opportunities for control.

In the summer of 2007, distinctive yellow leaf spots were observed for the first time in sugar beet in the Netherlands. Every year since then, infestation of fields has been reported and samples have been sent to the diagnostic service of the IRS (Institute of Sugar beet Research, Bergen op Zoom, NL). At the beginning (2007) yellow leaf spots were mainly reported from the North eastern region on sandy soils. In the following years, a rapid spread over all regions of the Netherlands was reported.

The first symptoms appear in June-August on the leaves of sugar beet and are characterised by small 0.5–2.0 mm, irregular, yellow spots on the leaves (Fig. 1). Subsequently, the yellow spots become necrotic, starting from the inside out, with the tissue turning brown (Fig. 2), and grow to become brown spots of 1–3 cm (Fig. 3). Conidia from the initial spots spread over neighbouring leaves and the whole plant becomes infected (Fig. 4). In cases of severe infestation, the heavily infested leaves die and new yellow spots appear on the newly formed leaves. Due to the loss of leaves the canopy declines and the soil becomes visible in August-September. Often, the infestation starts in patches and spreads over the whole field. After the first appearance research on the causal agent was initiated to confirm whether the yellow leaf spot disease was caused by viral, bacterial or fungal infection or nutrient deficiency. Subsequently, research was conducted to find management tools to prevent sugar yield loss due to this yellow spot disease.

Material and methods

Bacterial and viral causes

On four fields with symptoms of the yellow spot disease (IRS 07–161 and 07–163 Zwiggelte, NL and 07–164 and 07–165 Borger Compagnie, NL), leaves were sampled and 10 kg of soil was collected as randomly distributed subsample cores of bulk soil in and between the sugar beet rows taken with a 1.5 cm diameter auger (0–25 cm deep). From each field leaves and soil were collected in parts of the field showing a high degree of yellow leaf spots and parts with either no symptoms or the lowest degree of symptoms in that particular field. To test for a bacterial or viral cause, 4 weeks old sugar beet plants (cultivar Coyote, SESVanderHave N.V., Tienen, Belgium) were inoculated after light rubbing with a scourer, with leaf extract (sap inoculation) derived from the yellow spot infected leaves. In a second assay, sugar beet (cultivar Coyote) was sown in soils derived from infested fields. Both assays were conducted in a climate room [23 °C for 16 h in light conditions (20,000 lux) and 16 °C for 8 h in the dark] at the IRS (Bergen op Zoom, NL.).

Beside the assays in the climate room, as described above, extracts of naturally infected leaves showing severe symptoms or leaves showing no symptoms, sampled from the same fields were examined for the known sugar beet pathogenic viruses: BtMV (*Beet Mosaic Virus*), BYV (*Beet Yellows Virus*), BMYV (*Beet Mild Yellowing Virus*) and BNYVV (*Beet Necrotic Yellow Vein Virus*) with DAS-ELISA (Clark and Adams 1977) using the antibodies and positive control as provided by

Fig. 1 First symptoms of the yellow spot disease on sugar beet leaves, characteristic small (0.5–2.0 mm), irregular, yellow spots

Fig. 2 The yellow spots on sugar beet leaves necrotise from inside out into a brownish tissue, magnification 100×

Fig. 4 From the initial spots on sugar beet leaves, the symptoms spread over neighbouring leaves

Loewe Biochemica GmbH (Sauerlach, D). Infected leaves with various stages of symptom development were sent to the National Plant Protection Organisation (NVWA, Wageningen, the Netherlands) and the Pflanzenschutzamt of Nordrhein-Westfalen (Bonn, Germany) for electron microscope investigation for viral infection. The NVWA also performed a bio-assay on sugar beet, red beet and *Chenopodium* spp. inoculated with leaf extracts.

Identification of fungal isolates

To isolate the fungi growing in the leaf spots, small yellow spots were cut out of the leaves and the tissue sections (1 × 1 mm) were placed on plates of water agar medium amended with 1.2 % Chloramphenicol and 250 ppm Metalaxyl (WAcm) (Technical Agar no 3; Oxoid Ltd, UK). Where sporulation was observed under the binocular microscope (12.5–144×) on yellow spots with a small necrotised (brown) centre, conidia were isolated with 10 μl sterile distilled water. The water was pipetted onto the leaf spot, mixed by pipetting and transferred to either plates with WAcm medium and spread out with a sterile Drigalski spatula or onto a microscope glass slide. The conidia were examined in water under a microscope (Olympus CK 2, Olympus Corporation, Tokyo, Japan) and the size measured. The plates with tissue sections or conidia were stored in the dark at room temperature. When fungal mycelial growth was observed on the plates under the microscope (after 7–10 days), a plug of medium with mycelium was transferred after visual examination under the microscope to a new WAcm plate. Plates were stored for another 7 days in the dark at room temperature, visually examined under the microscope and a plug of medium with mycelium was transferred to plates with potato dextrose agar (PDA; Merck, Darmstadt, Germany). From each isolate single conidial isolates were produced and grown on PDA to obtain a pure culture. For DNA isolation the fungal isolates were transferred to potato dextrose broth (PDB; Difco Laboratories, Detroit USA) and grown for 10 days at 21 °C in the dark. The mycelium was collected with a sterile spatula, freeze dried and ground with a pestle. DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) using the protocol for purification of total DNA from plant tissue. The DNA was eluted in AE buffer (10 mM Tris-Cl, 0.5 mM EDTA (Qiagen)) and stored at −20 °C until further use.

Fig. 3 The later stage of symptoms on sugar beet leaves is necrotised tissue forming brown spots of 1–3 cm

A PCR was conducted using the ITS 2 and 4 primers (White et al. 1990) and a Taq PCR Master Mix Kit (Qiagen) with 5 ng DNA, 0.2 μM primer, 2.5 mM $MgCl_2$ and amplification at 57 °C (35 cycles). A second PCR was conducted using part of the glutaraldehyde-3-phosphate-dehydrogenase gene (gdp) with primers and methodology provided by Köhl et al. (2009). The amplicon was purified before sequencing with the Wizard DNA Clean-Up System (Promega; Madison, USA). Sequencing was done by Macrogen Europe (Amsterdam, the Netherlands). Sequences were compared to sequences in GenBank (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm for identification based on sequence homology.

Cultures of two isolates GV 10-140a1 (Dedemsvaart, NL) and GV 11-355a1 (Hijken, NL) were sent to the CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands). The obtained sequences of ITS, gdp and EF-1 alpha were compared with the *Stemphylium* sp. and isolates published in previous phylogenetic analyses (Câmara et al. 2002; Inderbitzin et al. 2009).

Climate room inoculation trials

To reproduce the symptoms on sugar beet under controlled conditions [23 °C for 16 h in light (20,000 lux) and 16 °C for 8 h in the dark], two independent climate room trials were conducted from September to November 2011 and from December 2011 to February 2012. For both trials, sugar beet seeds (cultivar Coyote; SESVanderHave, Belgium) were sown in sterile sand (sand from the river Maas; Vriends de Schelde BV, Bergen op Zoom, NL) and 10 day-old seedlings were transplanted to growing tubes (Teku Ø 5 cm, height of 18 cm) filled with a mixture of sterilised sand (sand from the river Maas; Vriends de Schelde BV, Bergen op Zoom, NL) and potting soil (Primasta Flower Power, Primasta BV, Asten, NL) in 9:1 ratio (*w/w*). Nutrients were added to the sand mixture as 1 g/kg slow release Osmocote Exact Mini (NPK 16-8-11+2 MgO+Trace Elements; Everris, ICL specialty fertilizers, Tel Aviv, IL). This mixture is called standard soil in the following text. Treatments were standard soil (pH=6.3), standard soil (pH=6.3) with beet cyst nematode (*Heterodera schachtii*) larvae and standard soil with low pH (first trial pH=4.8; second trial pH=4.2). The beet cyst larvae (J2) were added 4 weeks after seedlings were transplanted (first trial ca. 1630 larvae/tube; second trial ca. 1860 larvae/tube). On the treatments with standard soil and standard soil+beet cyst nematodes, 3 g/kg Dolokal (5 % Mg; Sibelco Europe MineralsPlus, Maastricht, NL) was added to adjust the pH. The beet cyst nematode larvae were obtained from the standard reference population used at the IRS (Bergen op Zoom, NL). This population originates from Woensdrecht (NL; IRS 07-01-04.02) and were reared on oilseed rape (*Brassica napus* cultivar Ladoga; Limagrain, Saint-Beauzire, F).

Twenty tubes (one plant per tube) of each treatment were grouped together. The leaves of 10 plants were damaged (two strokes/leaf with a scourer), leaves of the remaining 10 plants were left undamaged, prior to inoculation with fungal conidial suspension. Trays were arranged randomly in the climate room.

Prior to inoculation, a conidial suspension was produced as described by Köhl et al. (2009). Inoculation with fungal conidial suspension took place 8 weeks after seedlings were transplanted with two isolates of *Stemphylium* (GV 10-140a1; Dedemsvaart, NL and GV 11-265a; Langenboom, NL) and two isolates of *Alternaria alternata* (GV 10–187; Kloosterzande, NL and GV 10-234b1; Eerste Exloërmond, NL) or sterile tap water as a control. Isolates were obtained from sugar beet leaves infected with yellow leaf spot. Both *Stemphylium* sp. isolates belong to the largest group based on the ITS-sequence (see results section on identification). Each tray with twenty plants was sprayed with 5 ml of conidial suspension, the control with 5 ml sterile tap water. In the first trial, the densities of conidial suspensions were 6.9×10^4, 4.2×10^5, 1.7×10^5 and 7.5×10^5 conidia ml^{-1} for the isolates GV 10-140a1, GV 11-265a, GV 10–187 and GV 10-234b1, respectively. In the second trial, the conidia densities were 1.1×10^5, 1.1×10^5, 1.3×10^5 and 1.4×10^5 conidia ml^{-1}, respectively. Conidia density was determined in 2×10 μl of the conidial suspension using a haemocytometer (Bürker Türk; Marienfield; Lauda-Königshofen, D). Plants were covered with a transparent plastic bag (LPDE; 60×80 cm, 50 μm) directly after inoculation. Three days post inoculation plastic bags were removed with a final symptom assessment seven days post inoculation. When spots were visible the fungi were isolated and compared with the isolate used for inoculation.

Nutrient deficiency

For the investigation of nutrient deficiency, a field trial in a completely randomised block design with four

replications was conducted in Hooghalen (the Netherlands) in 2008. Plots (11×3 m, gross size; 9×3 m net size) were treated when the first yellow spots appeared. Treatments were: untreated, magnesium (25 kg/ha EPSO TOP, 16 % MgO and 32 % SO_3; K&S Kali GmbH, Kassel, Germany), manganese (1.5 l/ha Top Trace $Mn(NO_3)_2$; Cebeco Meststoffen BV, Rotterdam, the Netherlands) and UAN (25 kg/ha; 50 % ammonium nitrate and 50 % urea, Yara International ASA, Oslo, Norway). After 2 weeks the application was repeated. Plots were visually scored on the degree of symptoms (yellow and brown spots) on a scale from 0 (severely affected, sugar beet canopy died completely) to 10 (no symptoms, sugar beet canopy appeared healthy).

Fungicide field trials

Five fungicide field trials were conducted in the period 2008–2012. Trials were located in the Netherlands: Hooghalen (2008), Hijken (2010), Hijken (2011), Nieuw Buinen (2012) and Eerste Exloërmond (2012). Plots were 11 m×3 m (6×0.5 m rows) with a net size of 9 m×3 m in 2008 and 2010 and 15 m×3 m (net size 12.5 m×3 m) in 2011 and 2012. All trial fields had four replications in a completely randomised block design. The fungicides and dosages used on the different field trials are shown in Table 2. All field trials, except those in 2012 had two applications of fungicides with an interval of 3 weeks. The field trials in 2012 had three applications of the fungicides with an interval of 3 weeks. Just before harvest all the plots were visually scored on the degree of symptoms (yellow and brown spots) on a scale from 0 (severely affected, sugar beet canopy died completely) to 10 (no symptoms, sugar beet canopy appeared healthy).

Field trials were mechanically harvested with a sugar beet harvester (Franquet, Guignicourt, FR; adapted for field trial use by Leenpoel BV, Kamperland, NL). Gross weight was determined on the harvester in the field and three subsamples of ca. 20 kg were taken from each plot. Subsamples were analysed for sugar beet quality in the tare house of the IRS (Bergen op Zoom, NL). Beet brei samples were analysed for contents of sucrose and melassigenic compounds (sodium, potassium, and a-amino-N) following standard protocols (International Commission for Uniform Methods of Sugar Analysis 2007) in an automatic beet laboratory system (Venema, Groningen, NL). The field trial data were analysed with ANOVA using the statistical package GenStat (16th Edition) (Payne et al. 2013).

Host range

To test plant species for host status, 20 plants of seven crop species [red beet, *Beta vulgaris* cultivar: Loki (Horti Tops; Tuinplus Service, Heerenveen, NL); spinach, *Spinacia oleracea* cultivar: Nores (Horti Tops; Tuinplus Service, Heerenveen, NL); onion, *Allium cepa* cultivar: Stuttgarter Riesen (TOP Onions BV, s-Gravenpolder, NL); potato, *Solanum tuberosum* cultivars: Agria (Agrico UA, Emmeloord, NL), Rivièra (Agrico UA, Emmeloord, NL), Seresta (Sloots Agri B.V., Eenrum, NL) and Festien (Averis Seeds B.V. Veendam, NL); oilseed radish, *Raphanus sativus* subsp. *oleiferus* cultivar: Corporal (Innoseeds, Kapelle, NL); white mustard, *Sinapsis alba* cultivar: Gisilba (Kruse Saatzucht, Münster, D); annual ryegrass, *Lolium multiflorum* cultivar: Mont Blanc (Limagrain, Saint-Beauzire, F)] and two weed species black nightshade (*Solanum nigrum*) and fat hen (*Chenopodium album*) (Herbiseed, Twyford, UK) were grown in a climate room under the same conditions and in the same standard soil as described in the section on 'climate room inoculation trials'. Seeds were sown in sterile sand and 10 day-old seedlings were transplanted to growing tubes (Teku Ø 5 cm, height of 18 cm) filled with a mixture of sterilised sand and potting soil (the same as the standard soil in the climate room inoculation trials). The potato tubers (whole tubers; 25–28 mm) were directly planted in the tubes. Nutrients were added to the soil mixture as 1 g/kg slow release Osmocote Exact Mini (NPK 16-8-11+2 MgO+Trace Elements; Everris, ICL specialty fertilizers, Tel Aviv, IL) and 3 g/kg Dolokal (5 % Mg) was added to adjust pH. Twenty tubes (one plant per tube) of each plant species were grouped together in a tray. Trays were randomly placed in the climate room. Prior to inoculation, conidial suspension was produced as described for the climate room inoculation trials for sugar beet, using *Stemphylium* sp. isolate GV 11-196a1 (Blijham, NL isolated from infested sugar beet leaves) or sterile tap water as a control. Each tray was sprayed with 5 ml of conidial suspension or 5 ml sterile tap water with a hand-held sprayer. The *Stemphylium* sp. isolated belongs to the largest group based on the ITS-sequence (see results section on identification). The density of the conidial suspensions was 1.4×10^5 conidia ml^{-1}. Conidia density was determined in 2×10 μl of the conidial

suspension using a haemocytometer (Bürker Türk; Marienfield; Lauda-Königshofen, D). Plants were covered with a transparent plastic bag (LPDE; 60×80 cm, 50 μm) directly after inoculation. Seven days post inoculation plastic bags were removed and symptoms were assessed. In cases where spots were visible the fungus was isolated and compared with the isolate used for inoculation.

Results

Bacterial and viral causes and nutrient deficiency

The different investigations in bio-assays, ELISA and electron microscopy, showed no evidence that the yellow leaf spots in sugar beets were caused by either bacterial or viral infection. In addition the field trial for nutrient deficiency showed no significant differences between the nutrient applications and the untreated control (P=0.723). All the treatments suffered from the same degree of symptoms (data not shown).

Identification of fungal isolates

Stemphylium sp. was isolated from infected leaves collected from 24 fields. From each field, one isolate was used. Examination of the conidia and sporulating cultures under the microscope revealed that those isolates belonged to the *Stemphylium*-genus. On a microscope glass slide, in sterile water the size of the conidia was 31.6 (min. 14.8 – max. 49.9)×20.2 (min.14.8 – max. 29.5) μm. They had 1–5 distinct transverse and 1–3 longitudinal septa (Fig. 5) Based on the ITS-sequence, 22 of them were 100 % identical to each other. The other two also had an identical ITS-sequence. In between the two groups of isolates, the degree of homology between the ITS-sequence was 99.1 %. A search in the NCBI database using the BLAST algorithm resulted in the highest homology for the 22 isolates with *Stemphylium globuliferum* (GenBank accession: GU934566: identity 99 %; coverage 97 %; Total Score 1038). The second best homology occurred with an entry of *Stemphylium solani* (GenBank accession: AF203448; identity 98 %; coverage 100 %; Total Score 1011).

From older spots (necrotised tissue) on leaves from 17 fields *Alternaria alternata* was isolated. Sequencing and BLAST search of the ITS-sequence gave a 100 % match with known *Alternaria alternata* sequences (GenBank accession: AF071346).

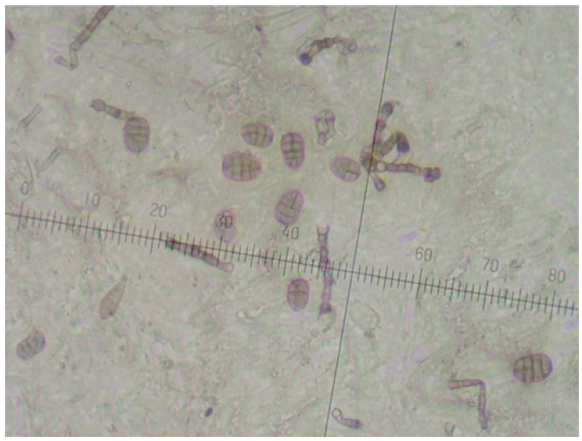

Fig. 5 Micrograph of *Stemphylium* sp. conidia from a yellow spot from sugar beet leaves at 200× magnification. Each dash on the bar represents 5 μm

The analyses at CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands) showed that isolate GV 11-355a1 (Hijken, NL), one of the group of two isolates with identical ITS sequence belongs to the *Pleospora herbarum* group (group C) provided by Inderbitzin et al. (2009). The isolate GV 10-140a1 (Dedemsvaart, NL), which belongs to the largest group of 22 isolates (with identical ITS sequence) was assigned to group E3 of the same study, containing only potentially cryptic and unnamed species.

Climate room inoculation trials

A fungal cause was confirmed by the two climate room inoculation trials (Table 1). In both independent trials, no leaf spots were observed in the control with sterilised tap water. For both *Alternaria alternata* isolates, no leaf spots were observed on undamaged leaves. Only when leaves were damaged, were a very few spots of *A. alternata* observed. All treatments inoculated with both *Stemphylium* isolates showed many leaf spots. For both *Stemphylium* isolates this was irrespective of leaf damage and the presence of nematodes or low pH of the soil. From the spots *Stemphylium* sp. was re-isolated. It was identical to the inoculated isolates.

Fungicide field trials

In the fungicide field trial of 2008, applications of Signum (boscalid and pyraclostrobin) significantly

Table 1 Results of two independent climate room trials with inoculation with spores of two *Stemphylium* sp. and two *Alternaria alternata* isolates obtained from sugar beet leaves infested with the yellow spot disease, on damaged and undamaged leaves of sugar beet (cultivar Coyote) grown without and with additional stress of *Heterodera schachtii* and low pH-KCl

Fungi	Isolate	Leaves	Additional stress factor	Leaf spots[a]
Control	Sterile tap water	undamaged	none	0
		damaged	none	0
Stemphylium	GV 10-140a1	undamaged	none	>101
		damaged	none	>101
Stemphylium	GV 11-265a	undamaged	none	21–100
		damaged	none	>101
A. alternata	GV 10-187	undamaged	none	0
		damaged	none	1–5
A. alternata	GV 10-234b1	undamaged	none	0
		damaged	none	1–5
Stemphylium	GV 10-140a1	undamaged	*H. schachtii*	21–100
		damaged	*H. schachtii*	21–100
Stemphylium	GV 11-265a	undamaged	*H. schachtii*	>101
		damaged	*H. schachtii*	>101
A. alternata	GV 10-187	undamaged	*H. schachtii*	0
		damaged	*H. schachtii*	1–5
A. alternata	GV 10-234b1	undamaged	*H. schachtii*	0
		damaged	*H. schachtii*	1–5
Stemphylium	GV 10-140a1	undamaged	low pH-KCl	>101
		damaged	low pH-KCl	>101
Stemphylium	GV 11-265a	undamaged	low pH-KCl	>101
		damaged	low pH-KCl	>101
A. alternata	GV 10-187	undamaged	low pH-KCl	0
		damaged	low pH-KCl	1–5
A. alternata	GV 10-234b1	undamaged	low pH-KCl	0
		damaged	low pH-KCl	0

[a] Average number of leaf spots per leaf grouped in the categories 0 (no spots visible), 1–5, 6–20 spots, 21–100 and>101 spots

($P<0.001$) reduced the foliar symptoms (Table 2). Also applications of Amistar Top (azoxystrobin and difenoconazole) reduced the amount of yellow leaf spots, although to a lesser extent compared to Signum. Both Opus Team (epoxiconazole and fenpropimorph) and Allegro (kresoxim-methyl and epoxiconazole) did not reduce symptoms significantly, with an equal infestation with yellow leaf spots and sugar yield compared to the untreated control. Sugar yield was highest with applications of Signum. Despite some reduction of symptoms, the Amistar Top treatments did not result in a significantly higher sugar yield compared to the untreated control, or the treatments with Opus Team and Allegro.

This pattern was repeated in the fungicide field trials of the subsequent years. In 2010 two applications of 0.35 l ha^{-1} with Sphere SC (trifloxystrobin and cyproconazole) had no effect on the yellow leaf spots and no effect on sugar yield, whereas the application of either of the boscalid containing fungicides [Signum and Venture (boscalid and epoxiconazole)] did. They both significantly reduced ($P<0.001$) the number of yellow leaf spots and these two treatments resulted in a significantly higher ($P=0.07$) sugar yield compared to untreated. In addition in both field trials in 2012, no effect on the amount of yellow leaf spots of the treatment with Opus Team (epoxiconazole and fenpropimorph) was found. Three applications of the fungicides Sphere SC and Spyrale EC (difenoconazole and fenpropidin) had a significant effect ($P<0.001$) on the reduction of the amount of yellow leaf spots and a significant higher sugar yield ($P<0.001$) compared to the untreated control. However, a significantly higher reduction ($P<0.001$) of yellow leaf spots was obtained with Retengo Plus (pyraclostrobin and epoxiconazole) in Nieuw Buinen (2012) compared to Sphere SC. Retengo Plus in 2012 reduced the number of yellow leaf spots significantly on both field trials ($P<0.001$) compared to Spyrale EC. In both field trials Retengo Plus had a significantly higher sugar yield ($P<0.001$) compared to both Spyrale EC and on the field trial in Nieuw Buinen a significantly higher ($P<0.001$) sugar yield compared to Sphere SC (Table 2). Only on the field trial in Nieuw Buinen did the application of Venture result in a significantly higher sugar yield ($P<0.001$) compared to Retengo Plus.

The field trial with low level of disease in 2011 at Hijken showed slightly different results. There was no difference in the number of yellow leaf spots ($P<0.001$) and sugar yield ($P<0.001$) for any of the fungicides applied. All the treatments had significantly better efficacy ($P<0.001$) and significantly higher sugar yield ($P<0.001$) compared to the untreated control. The untreated control gave a high sugar yield, 18.8 Mg ha^{-1} and all the fungicide treatments increased it significantly ($P<0.001$) to a maximum of 21.0 Mg ha^{-1}.

Table 2 Efficacy of fungicides against Stemphylium yellow leaf spot in sugar beet and resulting sugar yield (field trials on four locations in the Netherlands 2008–2012)

Name	Fungicide			Hooghalen 2008		Hijken 2010		Hijken 2011		Eerste Exloërmond 2012[f]		Nieuw Buinen 2012[f]	
	Authorisation holder	Active ingredient	Dosage per application (g a.i.[b] ha^{-1})	Efficacy (1-10)[d, e]	Sugar yield (Mg ha^{-1})[d]	Efficacy (1-10)[d, e]	Sugar yield (Mg ha^{-1})[d]	Efficacy (1-10)[d, e]	Sugar yield (Mg ha^{-1})[d]	Efficacy (1-10)[d, e]	Sugar yield (Mg ha^{-1})[d]	Efficacy (1-10)[d, e]	Sugar yield (Mg ha^{-1})[d]
Untreated	–	–	–	4.0^{A}	13.9^{A}	3.3^{A}	13.9^{A}	4.0^{A}	18.8^{A}	3.3^{A}	11.2^{A}	4.9^{A}	14.5^{A}
Opus Team	BASF Nederland B.V.	epoxiconazole	84.0	3.9^{A}	14.5^{A}					3.1^{A}	11.5^{A}	4.6^{A}	15.0^{A}
		fenpropimorph	250.0										
Allegro	BASF Nederland B.V.	kresoxim-methyl	93.8	4.3^{A}	13.7^{A}								
		epoxiconazole	93.8										
Retengo Plus[a]	BASF Nederland B.V.	pyraclostrobin	133.0							7.6^{C}	14.8^{C}	7.9^{CD}	16.7^{C}
		epoxiconazole	50.0										
Signum[a]	BASF Nederland B.V.	boscalid	133.5	8.3^{C}	16.3^{B}	7.5^{B}	14.9^{B}	8.1^{B}	20.3^{B}				
		pyraclostrobin	33.5										
Venture[a]	BASF Nederland B.V.	boscalid	302.9			7.8^{B}	15.0^{B}			8.9^{C}	15.9^{C}	8.9^{D}	17.7^{D}
		epoxiconazole	87.1										
Sphere SC	Bayer Crop-Science SA-N.V.	trifloxystrobin	131.3[c]			4.0^{A}	14.2^{AB}			5.5^{B}	13.0^{B}	6.8^{BC}	16.3^{BC}
		cyproconazole	56.0[c]										
Spyrale EC	Syngenta Crop Protection B.V.	difenoconazole	100.0					8.5^{B}	21.0^{B}	6.5^{BC}	13.3^{B}	6.4^{B}	15.8^{B}
		fenpropidin	375.0										
Score EC	Syngenta Crop Protection B.V.	difenoconazole	100.0					7.8^{B}	20.4^{B}				
Amistar Top[a]	Syngenta Crop Protection B.V.	azoxystrobin	200.0	5.9^{B}	14.4^{A}			8.2^{B}	20.5^{B}				
		difenoconazole	125.0										
Probability				< 0.001	< 0.001	< 0.001	0.07	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
LSD 5 %				0.9	1.23	0.9	0.88	1.1	1.21	1.3	1.12	1.4	0.55

[a] This product is not registered for fungicide use in sugar beet in the Netherlands

[b] *a.i.*, active ingredient

[c] Dosage per application of trifloxystrobin and cyproconazole used in 2012 was 93.8 and 40.0 g a.i. ha^{-1}

[d] Different letters in capitals in the superscript indicate significant differences (P>0.05)

[e] Efficacy scale 1 (very poor, leaves completely dead) – 10 (excellent, leaves completely healthy)

[f] In 2012 three fungicide applications were conducted, in the other years there were two

Host range

In the trial for the putative host range, spots were recorded and *Stemphylium* was re-isolated from those spots of potato (all cultivars), red beet, spinach, white mustard and fat hen. On the leaves of annual rye and black nightshade not a single spot could be detected. Onion and oil radish showed no obvious spots and from these two plant species *Stemphylium* could not be re-isolated.

Discussion

This paper describes an example of the potential threat new pathogens can have on crop yield and thus on food security. In the case of a new pathogen, like *Stemphylium* in Dutch sugar beet production, it takes time to find effective fungicides and to optimise control strategies. In the meantime, the pathogen is able to build up inoculum and spread over a larger area.

The yellow leaf spot disease in sugar beet , with the typical symptoms described in this paper, is caused by *Stemphylium* sp. The results suggest that the isolated *Stemphylium* sp. could act as a primary pathogen in sugar beet. More precise identification of the two groups of isolates (based on the sequence) is in progress and urgently needed for a better understanding of the fungus involved. So far, this *Stemphylium* sp. shown in this paper to be acting as a primary pathogen of sugar beet, does not seem to be *Stemphylium botryosum*, which was reported from Japan. There it was detected in late July 1974, being restricted to the sugar beet plants adjacent to garlic fields where garlic tip blight was severe (Uchino et al. 1986). The authors mention that a high inoculum density from the garlic was required for infection of sugar beet leaves and that infection from diseased sugar beet leaves to healthy ones seldom occurred. They also describe large brown lesions, similar to the later stage found in the Netherlands, but do not mention the tiny (0.5–2 mm) yellow spots. Farr et al. (1989) considered *S. botryosum* as a saprophyte or occasional, weak pathogen of sugar beet. *Stemphylium botryosum* is pathogenic on spinach (*Spinacia oleracea*) (Koike et al. 2001; Everts and Armentrout 2001) which belongs to the family, *Amaranthaceae*, like sugar beet. It was found to be endophytic on fat hen (*Chenopodium album*), which is another member of this family (Aly et al. 2010). Future research on the *Stemphylium* sp. isolated from the yellow spots on sugar beet leaves in the Netherlands, should be conducted to verify whether isolates of *S. botryosum* are pathogenic on sugar beet and how they are phylogenetically related to each other. The *Stemphylium* sp. isolated from the yellow spots on sugar beet leaves is shown to be pathogenic on spinach in this study.

In a study on the endophytes of table beet, a *Stemphylium* sp. was found in beet leaves sampled in Argentina and suggested to be either a latent virulent, avirulent or hypovirulent pathogen (Larran et al. 2000). This finding is in contrast with the observed aggressiveness of the *Stemphylium* sp. in the Dutch sugar beet production fields. Here, it spreads rapidly from leaf to leaf, with tiny (0.5–2 mm), but obvious yellow spots. Also in the climate room trials (in the conditions described in the current study) inoculation with the *Stemphylium* isolates resulted in a rapid infection, even on the undamaged leaves of healthy growing sugar beet plants.

The best match for the ITS sequence of the largest group of isolates from the yellow leaf spots was obtained with *S. globuliferum*. Little is known about the hosts of this species. It has been reported as pathogenic on legumes (Farr et al. 1989), including subterranean clover (*Trifolium subterraneum* L.) (Barbetti 1985) and alfalfa (*Medicago sativa* L.) (Samac et al. 2013; Samac et al. 2014). However, it is also reported as an endophyte (Debbab et al. 2009; Aly et al. 2011). The analyses at CBS-KNAW could not identify the *Stemphylium* sp. from sugar beet leaves as *S. globuliferum*. However, some of the *S. globuliferum* isolates investigated by Câmara et al. (2002) showed high similarity with the isolates in group E of Inderbitzin et al. (2009). The group of 22 isolates of *Stemphylium* sp. from sugar beet leaves also belonged to group E. The same analyses could exclude the *Stemphylium* sp. as *Stemphylium solani* (Woudenberg and Meijer 2014).

Hosts of *S. solani* include shallot (*Allium cepa* var. *aggregatum* syn. *Allium ascalonicum*), potato, spinach and radish (*Raphanus sativus*) (Zheng et al. 2009). Those authors report 21 plant species being a host for *S. solani*. This host range shows high similarities with the hosts found in our climate room assay, although we did not test all the 21 plant species by Zheng et al. (2009). In this first test we selected plant species commonly found in the crop rotations of the heaviest infested region of the Netherlands. Potato production is extremely dominant on the sandy soils in the North

East (almost 50 %). Sugar beet makes up nearly 25 % of the crop rotation of the average farm in this region (Van Welsenes 1973) so almost every sugar beet crop has potato as a pre-crop. The potatoes in the climate room host range assay showed the most severe symptoms of all tested crops. This severe infection indicates that potato is an excellent host for the *Stemphylium* sp. which causes the yellow leaf spot disease in sugar beet. *Stemphylium* sp. was also isolated from volunteer potato plants growing in infected sugar beet fields and from potato plants from commercial potato fields. This confirms the host status of potato under natural conditions. However, the impact of *Stemphylium* sp. on potato yield might be hidden by the use of fungicides used to control *Alternaria solani* (Horsfield et al. 2010; Pasche et al. 2004). Further research on *Stemphylium* sp. in potato and the effect of the crop rotation on inoculum densities should be conducted, as well as the identification of the *Stemphylium* species causing the yellow spot disease. It is important to understand more of its biology and host range. This would help to enable the development of management options fitting into the principles of integrated pest management (IPM), which is nowadays a requirement of crop production inside the European Union (Directive 2009/128/EC 2009). This identification work is still in progress.

The results from the fungicide field trials illustrate that with the currently registered fungicides (Table 2), it is hard to obtain an effective control of *Stemphylium* sp. causing the yellow leaf spot disease without any yield loss. The fungicides registered in the Netherlands have little or no effect on the amount of yellow leaf spot and sugar yield. This is a likely explanation for the rapid spread of the yellow leaf spot disease in Dutch sugar beet production, from a small, restricted area in 2007 to more than 23.000 ha in 2012 all over the Netherlands.

Different fungicides with active ingredients belonging to the same group or class as sorted by the Fungicide Resistance Action Committee (FRAC 2014) have a different efficacy. For instance Opus Team (epoxiconazole) and Spyrale (difenoconazole) and the triazole and strobilurin containing Sphere (trifloxystrobin and cyproconazole) and Retengo Plus (pyraclostrobin and epoxiconazole) have shown different efficacy towards *Stemphylium* sp. in this research. This is also observed in sugar beet for the well investigated foliar pathogen *Cercospora beticola* (Karaoglanidis and Thanassoulopoulos 2003; Gado 2007; Bolton et al. 2012). However, despite those differences in sensitivity towards fungicides *Cercospora beticola* still can be managed by alternating fungicides with active ingredients belonging to different groups (Bolton et al. 2013; Karaoglanidis and Bardas 2006; Secor et al. 2010). This indicates that the *Stemphylium* sp. causing yellow leaf spot can also be managed once fungicides with a high degree of efficacy are registered. To prevent unnecessary use of fungicides further research will be conducted to incorporate *Stemphylium* sp. into the supervised control of foliar diseases in Dutch sugar beet production (Vereijssen et al. 2007).

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