Biological characterization of fenpicoxamid, a new fungicide with utility in cereals and other crops

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Abstract

BACKGROUND: The development of novel highly efficacious fungicides that lack cross-resistance is extremely desirable. Fenpicoxamid (Inatreq™ active) possesses these characteristics and is a member of a novel picolinamide class of fungicides derived from the antifungal natural product UK-2A.

RESULTS: Fenpicoxamid strongly inhibited in vitro growth of several ascomycete fungi, including Zymoseptoria tritici (EC50, 0.051 mg L−1). Fenpicoxamid was converted by Z. tritici to UK-2A, a 15-fold stronger inhibitor of Z. tritici growth (EC50, 0.0033 mg L−1). Strong fungicidal activity of fenpicoxamid against driver cereal diseases was confirmed in greenhouse tests, where activity on Z. tritici and Puccinia triticina matched that of fluxapyroxad. Due to its novel target site (Qi site of the respiratory cyt bc1 complex) for the cereals market, fenpicoxamid is not cross-resistant to Z. tritici isolates resistant to strobilurin and/orazole fungicides. Across multiple European field trials Z. tritici was strongly controlled (mean, 82%) by 100 g as ha−1 applications of fenpicoxamid, which demonstrated excellent residual activity.

CONCLUSIONS: The novel chemistry and biochemical target site of fenpicoxamid as well as its lack of cross-resistance and strong efficacy against Z. tritici and other pathogens highlight the importance of fenpicoxamid as a new tool for controlling plant pathogenic fungi.

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Keywords: fenpicoxamid; Inatreq™ active; UK-2A; Zymoseptoria tritici; Mycosphaerella graminicola; Qi site; fungicide resistance management

1 INTRODUCTION

In order to protect both the quality and yield of agricultural and horticultural crops, growers must prevent or minimize damage and losses resulting from diseases initiated by infection(s) by plant pathogenic fungi. Fungicides representing a number of different chemistry classes and modes of action are used to prevent or control the onset of disease so as to maintain a healthy crop. However, many fungi have a propensity to develop resistance to fungicides. The risk for this occurring is determined by multiple factors including pathogen biology, the timing and frequency of fungicide use, whether they are single- or multi-site inhibitors used alone or in mixtures or in alternation with chemistries of different modes of action etc., and whether or not the resistance mechanism confers a competitive fitness penalty to resistant phenotypes.1−5 For example, over recent years in the European cereals market Zymoseptoria tritici (synonym Mycosphaerella graminicola, wheat leaf blotch), the pathogen of greatest concern in terms of yield losses in winter wheat production,6 has developed widespread resistance to the strobilurin class of fungicides7−12 and substantial sensitivity loss to theazole chemistries.13−18 In the case of the strobilurins, resistance is predominantly the result of a single point mutation, G143A, in the Qo binding domain of the mitochondrial cyt bc1 complex7,12 which dramatically reduces binding and confers a qualitative resistance with little or no fitness penalty. Azole fungicide resistance in Z. tritici, on the other hand, is quantitative in nature, with the appearance of different point mutations in the CYP51 gene (which encodes the ergosterol 14-C demethylase target enzyme) across less sensitive isolates, some of which may contain multiple such mutations with little evidence of any fitness loss.19−22 Additionally, some add-in strains have been identified that can overexpress CYP5113 and/or the efflux pump MgMFS123 due to promoter inserts in the corresponding genes.

The strobilurin and azole fungicides have played a prominent role in multiple crop markets for many years, but the emergence and spread, throughout much of the European cereals market, of Z. tritici resistance to these chemistry classes established a critical need for new products with novel modes of action for this market.

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in particular. The agrochemicals industry responded to this need with the development and introduction of new carboxamide (succinate dehydrogenase inhibitor, SDHI) chemistries, which inhibit the succinate dehydrogenase (complex II) of the mitochondrial respiratory chain. Although not a new mode of action (carboxin inhibits complex II also) per se, it was novel to the cereals foliar fungicide market and BASF’s introduction of boscalid, primarily for eye spot control, in 2003 was later (2010–2012) followed by the more efficacious next generation active ingredients fluxapyroxad (BASF Crop Protection), bixafen (Bayer CropScience), penthiopyrad (DuPont Crop Protection) and isopyrazam (Syngenta), and then most recently (2016) by benzovindiflupyr (Syngenta). The rapidly expanding use of these SDHI chemistries, especially for Z. tritici control in cereals, potentially establishes a high level of selection pressure and associated resistance risk, particularly since existing strobilurins or azoles with which they are co-formulated as mixture products have diminishing efficacy. Indeed, the detection of SDHI resistant isolates of Z. tritici obtained from sites in Ireland24 and the UK [https://cereals.ahdb.org.uk/press/2016/february/12/sdhi-fungicide-sensitivity-shift-detected-in-two-uk-septoria-isolates (accessed 29 September 2016)] has recently been reported. Furthermore, isolates of Pyrenophora teres (net blotch of barley) resistant to SDHI fungicides have also been detected in several European cereal growing geographies.25 Cases such as these serve as a constant reminder of the need for continued innovation towards identifying new active chemistries with novel modes of action.

With this in mind, the fungicide discovery research program at Dow AgroSciences LLC, in collaboration with Meiji Seika Pharma Co. Ltd, identified fenpicocoxamid (Inatrex™ active; Fig. 1) as a novel picolinamide fungiclude currently under development for use primarily in cereals but also in other crops. Fenpicocoxamid is an acyloxymethyl ether derivative of the natural antifungal compound UK-2A (Fig. 1) originally isolated from fermentation broths of the actinomycete Streptomyces sp. 517–02,26–29 extracts of which demonstrated strong antifungal activity against a broad spectrum of fungi in vitro assays.28 Picolinamide chemistry delivers a novel biochemical mode of action for the cereals fungicide market involving inhibition of mitochondrial complex III via binding to the Q, ubiquinone binding site20 rather than to the Qo site targeted by the strobilurin class of fungicides and, as such, no target-site-based cross-resistance to strobilurin fungicides would be anticipated (D. Young et al., manuscript in preparation). Prior validation of the bc1 Q, binding site as a commercially viable fungiclude mode of action was provided by cyazofamid (ISK Biosciences Corp.), a structurally unrelated chemistry which uniquely binds to the Q, site of oomycete pathogens only.31

Through a collaborative effort between Dow AgroSciences LLC and Meiji Seika Pharma Co. Ltd, the attributes of UK-2A natural product were more extensively characterized, including greenhouse and field efficacy; spectrum against agriculturally relevant plant pathogens, physical properties and in planta stability. However, translation of the highly compelling target site and in vitro fungal growth inhibition by UK-2A to strong greenhouse and field fungicidal activity against key fungal diseases was not fully realized. UK-2A, nonetheless, was considered an attractive candidate for semi-synthetic modification and optimization of antifungal activity and other key attributes. Of the many analogs prepared, the isobutyryloxymethyl ether, produced as a one step post-fermentation modification of the picolinamide OH group of UK-2A, was identified as the first development candidate. The present paper will summarize the biological characterization and associated attributes of fenpicocoxamid, including its ability to provide strong control of isolates of Z. tritici resistant to current classes of cereal fungicides. The supplementary information provides a synopsis of the physical, environmental and toxicological properties of fenpicocoxamid that are key to product registration.

2 MATERIALS AND METHODS

2.1 Test compounds

UK-2A was produced by Meiji Seika Pharma Co. Ltd, Tokyo, Japan. UK-2A methyl ether (Fig. 1, R1 = CH3), fenpicocoxamid ([[4-methoxy-2-[[[3S,7R,8R,9S]9-methyl-8-(2-methyl-1-oxopropoxy)-2,6-dioxo-7-(phenylmethyl)-1,5-dioxonan-3-yl]amino]carbonyl]-3-pyridinyl][oxy]methyl-2-methylpropanoate, ISO 1750 (provisionally approved). The four plant pathogens Z. tritici, Leptosphaeria nodorum, Pyricularia oryzae and Ustilago maydis were used to evaluate and compare the inherent antifungal activity of fenpicocoxamid, UK-2A, UK-2A methyl ether and several commercial fungicides. Fenpicocoxamid and UK-2A were additionally tested against the oomycete pathogen Phytophthora infestans. The 96-well microtiter plate based growth inhibition assays employed for Z. tritici, L. nodorum, P. oryzae and U. maydis were essentially as described previously by Owen et al.,32 with minor modifications. The growth medium used for P. infestans assays was a synthetic medium consisting of 0.5 g NaNO3, 0.36 g MgCl2, 6H2O, 0.2 g KH2PO4, 0.2 g CaCl2, 50.0 mg (NH4)2SO4, 13.2 mg ferric-sodium ethylenediaminetetraacetic acid (FeNa-EDTA), 0.4 g α-ketogluconate, 0.2 g succinic acid, 10.0 g dextrose, 4.0 g casamino acids and 0.2 mg thiamine L−1, adjusted to pH 5.5. Inoculum was prepared by flooding mycelium growing on rye seed agar33 plates with the above medium and scraping with a spatula to release sporangia and zoospores. Initial inoculum densities were 2.0 × 104 spores mL−1 for U. maydis, 4.0 × 105 spores mL−1 for P. oryzae and P. infestans, 1.0 × 108 spores mL−1 for Z. tritici and 2.0 × 105 spores mL−1 for L. nodorum.

Figure 1. Structures of (A) fenpicocoxamid and (B) UK-2A (R1 = H). CAS registration number 517875-34-2. ©2017 The Authors. Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.
A 3-fold dilution series of test compounds was prepared using stock solutions in dimethyl sulfoxide (DMSO) and 2 μL aliquots were added to the wells to deliver a total of 12 test concentrations ranging from 0.000011 mg L⁻¹ to 2 mg L⁻¹ for fenpicoxamid, UK-2A and commercial standards, and from 0.000102 mg L⁻¹ to 18 mg L⁻¹ for UK-2A methyl ether. Test plates were incubated in the dark for 48 h (P. infestans, U. maydis) or 72 h (P. oryzae, L. nodorum and Z. tritici) at 20 °C (P. infestans), 22 °C (P. oryzae, Z. tritici and L. nodorum) or 24 °C (U. maydis), and initial and final cell density readings were determined using a NepheloStar nephelometer (BMG Labtech GmbH, D-77799 Ortenberg, Germany). Assays were run in triplicate and percentage growth inhibition was calculated by reference to control wells containing only growth media, amended with 2 μL DMSO, and inoculum. Fungicide sensitivities were determined as 50% effective concentration (EC₅₀) using a dose–response relationship provided by a two-parameter logistic model within the statistical software R (RProj) in conjunction with the Ritz and Streibig dose–response curve (drc) package. 34

2.3 Greenhouse evaluation of fenpicoxamid

2.3.1 Plant material and pathogen inoculation

Host plants used for greenhouse testing of fungicide activity against wheat pathogens Z. tritici (wheat leaf blotch), Puccinia triticina (wheat leaf rust) and L. nodorum (glume blight), Rhyhchosporium secalis (barley scald), Colletotrichum lagenarium (cucumber anthracnose), Alternaria solani (tomato early blight), Cercospora beticola (leaf spot of sugar beet), Venturia inaequalis (apple scab) and P. oryzae (rice blast) were wheat (cv. Yuma), barley (cv. Harlington), cucumber (var. Bush Pickle), tomato (var. Outdoor Girl), sugar beet (var. HH88), apple (var. McIntosh) and rice (Japonica, cv M202). Seeds (8–12 per pot for wheat, barley and rice, 1 per pot for cucumber, tomato, apple and sugar beet) were planted into 27.5 cm² pots containing Metro-Mix® (Sun Gro Horticulture, Agawam, MA, USA) and grown in a greenhouse (16 h photoperiod at 20 °C) for 7–8 days (wheat and barley) or 12 – 14 days (cucumber, rice and tomato). Sugar beet plants were trimmed regularly to maintain a uniform plant size prior to testing, while apple seedlings were trimmed to leave only the two youngest leaves attached. Plants selected for testing were inoculated with an aqueous suspension of spores prepared from stock cultures maintained on agar plates (A. solani, C. lagenarium, L. nodorum, P. oryzae, R. secalis, Z. tritici) or by washing from infected leaves (C. beticola, V. inaequalis) and delivered as a fine mist using a DeVilbiss sprayer. Spore concentrations were adjusted to 1.0 × 10⁷ (L. nodorum, R. secalis, Z. tritici), 5.0 × 10⁶ (A. solani, C. lagenarium, P. oryzae, V. inaequalis) or (1.0–2.0) × 10⁶ (C. beticola) spores ml⁻¹ prior to addition of Tween 20 (final concentration 1 g L⁻¹) to facilitate leaf wetting and coverage. Inoculum of P. triticina was prepared by suspending harvestedurediospores in water with the aid of Tween 20, at a rate of 0.1 g urediospores and 3 drops Tween 20 per 100 ml. Spore suspensions were filtered through two layers of cheesecloth, and the filtrate was used to inoculate host plants as described above. Plants were inoculated either 3 days prior to or 1 day post fungicide application. Inoculated plants were subsequently kept in a dew chamber at 20–22 °C and 100% humidity for either 18–24 h (P. triticina), 48 h (A. solani, C. beticola, C. lagenarium, P. oryzae, R. secalis, V. inaequalis) or 72 h (L. nodorum, Z. tritici) to support spore germination and host leaf infection. Infected plants were then transferred either to greenhouses maintaining temperatures of 20 °C (L. nodorum, R. secalis, V. inaequalis, Z. tritici) or 24 °C (P. oryzae, P. triticina) or to a growth chamber set at 23 °C (A. solani, C. lagenarium) until disease symptoms were fully expressed on the first leaf of untreated plants (first two leaves in the case of A. solani on tomato). Sugar beet plants inoculated with C. beticola were also returned to a 24 °C greenhouse where they were maintained under a Perspex hood, with bottom ventilation, to deliver higher humidity levels. For all pathosystems, tests were carried out in triplicate and percentage disease control was subsequently calculated using the ratio of disease on treated plants relative to untreated plants. ED₅₀ or ED₉₀ values were determined from the resulting dose–response curves, using either the two-parameter logistic or four-parameter (Weibull) models contained within the R (RProj) or JMP Pro 11 statistical software, respectively.

2.3.2 Cross-resistance testing

Cross-resistance studies were carried out on four United Kingdom isolates (UK-4, UK-7, UK-12 and UK-13) of Z. tritici. UK-4 and UK-7 were isolated from lesions on infected wheat leaves collected at a Dow AgroSciences field trials location in Warwickshire in 2015, and their CYP51 genes were sequenced at Dow AgroSciences by C. Avila Adame. Isolates UK-12 and UK-13 were kindly provided by the Fraaije laboratory at Rothamsted Research (Harpenden, UK), where they are annotated as ADAS 14 80.4 and NIA 321.17, respectively. All four isolates carry target-site-based mutations conveying resistance to both triazole and strobilurin classes of fungicides. Isolate UK-12 also carries a 120 bp CYP51 promoter insert linked with CYP51 overexpression (B. Fraaije, personal communication). Additionally, both UK-12 and UK-13 have the 519 bp MgMFS1 promoter insert linked with overexpression of MgMFS1 involved in multi-drug resistance. Isolate ATCC 26518, used for the routine Z. tritici testing described above and which remains highly sensitive to both triazole and strobilurin fungicides, was included for comparison. Conditions for testing these isolates were otherwise identical to the general Z. tritici methodology described above.

2.3.3 Fungicide formulation and application

For efficacy testing on P. triticina and efficacy and cross-resistance testing on Z. tritici, fungicide applications were made at low spray volumes typical of field application practices. For this purpose an early stage EC formulation of fenpicoxamid was used along with commercial fungicide formulations Imtrex™ (fluxapyroxad, BASF Crop Protection), Ignite™ (epoxiconazole, BASF Crop Protection) and Headline™ (pyraclostrobin, Bayer CropScience) to prepare spray formulations in distilled water. Each fungicide was evaluated using rates of 100, 25, 6.25, 1.56 and 0.39 g as ha⁻¹. Formulated materials were applied to the plants with a spray volume of 200 L ha⁻¹ using a track sprayer (DeVries Manufacturing, Hollandale, MN, USA) equipped with a Tee Jet 8003E spray nozzle operating at 32 psi. The track speed was 1.8 mile h⁻¹, and the distance between nozzle and bench was 25 in. Treated plants were allowed to dry for 1 h prior to handling.

For tests involving high volume spray applications, technical material of fenpicoxamid, epoxiconazole and fluxapyroxad was dissolved in acetone, from which further serial dilutions were made. Final spray solutions were obtained by mixing aliquots of the acetone solutions with 9 volumes of water containing 110 ppm of Triton X-100 to deliver concentrations of 100, 25, 6.25, 1.56 and 0.39 mg L⁻¹. Applications to seedling plants were made to run off 24 h prior to inoculation, using an automated turntable sprayer (DeVries Manufacturing, Hollandale) fitted with two 6218-1/4JAUPM spray nozzles (Spraying Systems Co., Wheaton, IL, USA) and operated at 25 psi.
2.3.4 Bioassay mobility tests

Mobility bioassays were conducted using the *P. triticina/wheat pathosystem and compared fenpicoxamid, formulated as a 5% laboratory test EC, to epoxiconazole (Ignite™) as well as untreated controls. Fenpicoxamid and epoxiconazole were prepared as 500 mg as L⁻¹ application formulations of which 2 μL volumes were applied along a marked line (5 cm from the leaf tip) on the adaxial surface of primary leaves held in a horizontal position, each fungicide treatment being replicated six times. Plants were inoculated with *P. triticina* 1 day after compound applications and maintained as described above until disease was fully expressed on primary leaves of control plants. Photographic imaging was used to capture a visual record of treated leaves, or portions thereof, that were maintained in a disease-free state following drop-line application.

2.4 Field testing

The field data presented in this paper are based on 10 *Z. tritici* trials (two in Denmark, three in France, two in Germany and three in the UK) established in 2014 and 2015 in locations where wheat is grown commercially and where *Z. tritici* is prevalent. The trials were carried out by Dow AgroSciences and independent contractor companies and research institutes, all of which follow guidelines and standards established and maintained by the European and Mediterranean Plant Protection Organization (EPPO) [http://pp1.eppo.int/ (accessed 15 September 2016)] as well as the principles of good experimental practice. Fenpicoxamid was evaluated at several rates, prepared using a 50 g as L⁻¹ early stage EC formulation, in accordance with EPPO guidelines PP 1/026 v 4 (fungal and ear diseases on cereals) and PP 1/225 v 2 (minimum effective dose). However, for the purposes of illustrating fenpicoxamid’s field efficacy against *Z. tritici*, in this work only the data for 100 g as ha⁻¹, the proposed label rate for the EU, are presented. The reference treatment included was prothioconazole (Proline 275™, Bayer CropScience) which was applied at 0.72 L ha⁻¹ (198 g prothioconazole ha⁻¹). The trials were of a randomized complete block design with four replicates and a plot size ranging between 20 m² and 36 m². The varieties tested (cv. Akteur, J8 Asano, Mariboss, Pakito, Garcia, Dinosaur, Trapez, Conqueror and Cordiale) are all highly susceptible to *Z. tritici*. All treatments were applied using self-propelled, bicycle or knapsack precision plot sprayers equipped with conventional or low drift flat fan nozzles delivering water volumes of between 150 and 220 L ha⁻¹. Treatments involved a single application timing, usually at winter wheat growth stage 32-33 (second to third node detectable) based on the Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (BBCH) decimal scale, although one trial was treated at BBCH 37–39 (flag leaf just visible to flag leaf fully unrolled). Applications were generally protectant to leaf 3, typical for a commercial T1 fungicide and subjected to analysis of variance using the mixed model restricted maximum likelihood estimation, a method where treatment is modeled as a fixed effect and trials as a random effect. Data from across all 10 trials were subsequently combined to generate cross-trial mean percentage disease control values and subjected to Tukey’s HSD comparison to test for differences between treatments at the <0.05 probability level.

2.5 Microscopy studies

2.5.1 Culture preparation and leaf inoculation

Confocal microscopy of *Z. tritici* development on wheat leaf surfaces was facilitated by use of a GFP-*Z. tritici* strain (strain B3) kindly provided by the Fraaije laboratory at Rothamsted Research (Harpenden). A conidial suspension was created by removing spores from a 5-day-old culture growing on potato dextrose agar by surface irrigation with distilled water. Following enumeration with a hemacytometer, spore concentration was adjusted to 2 × 10⁵ conidia mL⁻¹ and Tween 20 was added to a final concentration of 1 g L⁻¹. Seedlings of wheat cv. Yuma, sprayed 24 h earlier with a 6.25 mg L⁻¹ high volume application of fenpicoxamid, formulated in 10% aqueous acetone containing 100 mg L⁻¹ Triton X-100, were subsequently inoculated to run off using a hand-operated spray bottle. Inoculated plants were incubated in a dark dew room overnight, then in an illuminated dew room for 24 h, followed by transfer to a greenhouse for a further 24 h prior to sampling.

For environmental scanning electron microscope (ESEM) imaging, spores of *Z. tritici* isolate ATCC 26518 were harvested from pycnidia, present on infected leaves of wheat seedlings (cv. Yuma) showing advanced disease symptoms, by soaking the leaf tissues in deionized water for 30 min. The inoculum spore concentration was adjusted to 1.0 × 10⁷ spores mL⁻¹ and Tween 20 was added to a final concentration of 0.005% v/v. Wheat seedlings sprayed with 6.25 mg L⁻¹ fenpicoxamid were inoculated with the spore suspension in prepared Petri dishes and maintained prior to ESEM imaging as described below.

2.5.2 Confocal microscopy

Primary leaves were removed from inoculated seedlings and transported to the microscopy laboratory in covered Petri dishes. Individual leaves were mounted on glass slides using double-sided tape and imaged dry, and without a coverslip, using a 10x objective on a Leica TCS-AOBS-SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Samples were illuminated with the 488 nm line of an argon laser, and emission was collected between 500 and 550 nm. Image stacks were collected, and maximum projection images were generated for analysis and publication.

2.5.3 ESEM

Primary leaf blades were excised from wheat seedlings 24 h after fenpicoxamid treatment and the cut ends were sandwiched between two layers of potato dextrose agar, the upper layers having been prepared from the middle section of agar using a scalpel (Fig. 2). This allowed the excised leaves to remain hydrated and green over the course of the experiment. Zones to receive inoculum (ca 1 μL droplets) were then delineated with a permanent marker to facilitate sample imaging. After inoculation, Petri plates were covered to prevent evaporation of the inoculum droplets, and spores were allowed to settle onto leaf surfaces for 1 h. Residual surface liquid was wicked away using a Kimwipe™ in order to avoid deposition of residues that accompanied complete evaporation and caused some obscuring of both surface leaf detail and spores. After 72 h, leaves were removed from the agar plates, mounted on a Peltier stage and imaged at 3 °C and 4.5 torr on a FEI
Quanta 250 ESEM (FEI, Hillsboro, OR, USA). Micrographs were manually colorized using the GNU Image Manipulation Program (GIMP, v2.8) to emphasize the fungal tissue on the surface of the leaves and to balance contrast between panels.

2.6 Surface stability studies

Surface stability studies using non-radiolabeled fenpicoxamid and UK-2A were carried out on both wheat leaves and filter paper discs. For wheat leaf studies, 8-day-old plants were sprayed to run off with 25 mg L\(^{-1}\) spray solutions (in 10% aqueous acetone containing 100 mg L\(^{-1}\) Triton X-100) as described above for high spray volume applications. At 1, 24 and 96 h after application, plants were sampled in triplicate by harvesting all aerial tissues. Surface deposits of fenpicoxamid or UK-2A were subsequently removed by two consecutive 1 min washes in 100% acetonitrile, and the washings were then combined. To recover compounds from within leaf tissue, the plants were subsequently ground in liquid N\(_2\) and sonicated for 2 min with 20 mL 100% acetonitrile, and the resulting extracts were centrifuged at 3000 g for 10 min. The pellets were extracted a second time and the two supernatants were combined. The washings and extracts were concentrated under N\(_2\) gas before reconstituting in 1 mL 100% acetonitrile for liquid chromatography–mass spectrometry analysis.

For filter paper studies, 200 mL of 50 μg mL\(^{-1}\) stock solutions of fenpicoxamid and UK-2A in acetonitrile were applied uniformly to discs of Whatman #42 filter paper. After drying, one set of discs was maintained in the dark, another under ambient laboratory light and a third set in a growth chamber maintaining a 20% relative humidity and a 16 h photoperiod (both visible and UV A/B wavelengths) and an average light intensity of 275 μW cm\(^{-2}\). Samples was removed after 0.5, 4 and 24 h, added to 10 mL acetonitrile and maintained overnight at 4 °C to facilitate thorough compound extraction and recovery.

Aliquots of samples generated in both studies were subsequently analyzed on a 5 μm Luna C\(_{18}\) (150 × 4.6 mm) column (Phenomenex, Torrance, CA, USA) using an Agilent 1260 LC system (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 6150 quadrupole mass spectrometer. Ejection conditions consisted of a linear gradient commencing at 50:50 v/v aqueous 1% acetic acid:acetonitrile, increasing to 100% acetonitrile at 15 min, which was maintained isocratically until 17 min before returning to 50:50 by 20 min, at a flow rate of 1.0 mL min\(^{-1}\). Mass spectral analysis was carried out using an electrospray source in positive ionization mode. A peak corresponding to each compound in the extracted ion chromatograms was used to quantify the recovery of the compound from samples using a calibration curve constructed from a dilution series prepared for each compound. All experiments were conducted in triplicate, unless otherwise indicated.

2.7 Metabolism of fenpicoxamid in *Z. tritici* and wheat cell suspension cultures

Cultures of *Z. tritici* isolate ATCC 26518 were prepared in potato dextrose broth (MP Biomedicals, Santa Ana, CA, USA), consisting of 24 g L\(^{-1}\) potato dextrose adjusted to pH 6.8, by addition of spores harvested from 3 day cultures grown on potato dextrose agar at 18 °C under black lights to give a final spore concentration of 1.0 × 10\(^6\) spores mL\(^{-1}\). Aliquots (10 mL) of the spore suspension were added to autoclaved 20 mL glass scintillation vials (Fisher Scientific, Waltham, MA, USA) and capped with sterilized foam Identi-Plugs (Jaece Industries Inc., North Tonawanda, NY, USA). Controls contained 10 mL potato dextrose broth without fungal spores. The control and inoculated tubes were incubated at 18 °C on an orbital shaker operating at 140 rpm for 48 h prior to addition of \(^{14}\)C-labeled fenpicoxamid.

Cell suspension cultures of wheat (*Triticum aestivum* L. cv. Anza) were prepared and grown as previously described.\(^{36,37}\) Aliquots (7 mL) of 7-day-old cell suspensions (cell density equivalent to 30 mg cells mL\(^{-1}\) or culture medium alone (Murashige and Skoog medium containing 2 mg L\(^{-1}\) 2,4-D\(^{38}\)) were dispensed into six-well culture plates and incubated on an orbital shaker at 130 rpm and 27 °C for 24 h before addition of \(^{14}\)C-labeled fenpicoxamid.

A 1 mg mL\(^{-1}\) stock solution of \(^{14}\)C-labeled fenpicoxamid in acetonitrile was prepared and, respectively, 14 and 20 μL aliquots were added to the 7 mL wheat and 10 mL *Z. tritici* cultures prepared as described above, to give a final fenpicoxamid concentration of 2 ppm (0.62 and 0.88 μCi per treatment for wheat and *Z. tritici*, respectively). A set of the samples was harvested immediately after fenpicoxamid addition, and the remainder were shaken on an orbital incubator at 140 rpm and 22 °C for 3 h until sampling. At each sampling time, aliquots (0.5 mL) of fungal or cell suspension cultures were mixed with 0.5 mL of acetonitrile and pulverized with 0.4 mm stainless steel (wheat cells) balls (Glen Mills Inc., Clifton, NJ, USA) at 1500 strokes min\(^{-1}\) for 3 min using a SPEX Geno/Grinder (SPEX, Metuchen, NJ, USA). The extracted samples were centrifuged at 10 000 g for 10 min, and the resulting supernatants were collected. The pellets were re-extracted with 1 mL acetonitrile and centrifuged. The supernatants resulting from the two extractions were combined for analysis. Radioactivity in these samples was determined by subjecting aliquots to liquid scintillation counting using a Beckman LS6000 scintillation counter (Beckman, Fullerton, CA, USA).

Aliquots of the extracts were initially characterized using the Agilent 1260 LC system, column and elution conditions described above, with the addition of a flow scintillation analyzer (Packard, Shelton, CT, USA) for monitoring radioactivity. Mass spectral analysis was subsequently carried out using an Agilent 1290 LC system equipped with an QTOF 5600 mass spectrometer (AB Sciex, Framingham, MA, USA) using a 4 μm Synergi Hydro RP (150 × 4.6 mm) column (Phenomenex, Torrance). Elution conditions consisted of a flow rate of 1.0 mL min\(^{-1}\) and a linear gradient of aqueous 0.1% formic acid:acetonitrile, commencing at 95:5 (v/v), increasing in acetonitrile content to 50:50 (v/v) at 30 min, then to 100% acetonitrile at 40 min which was maintained for a further 10 min prior to returning to the starting conditions. Mass spectral analysis was
carried out using an electrospray source operating in positive ionization mode to confirm the identity of fenpicoxamid and UK-2A.

3 RESULTS AND DISCUSSION

3.1 In vitro studies

3.1.1 Fungitoxicity

Fenpicoxamid was tested for inherent antifungal activity against a panel of plant pathogenic fungi, consisting of L. nodorum, P. infestans, P. oryzae, Z. tritici and U. maydis in comparison with UK-2A and a number of commercial fungicides as reference. The data generated are presented in Table 1 as EC50 values and their corresponding 95% confidence limits in parenthesis. Reference to Table 1 indicates that UK-2A is the most active compound against Z. tritici and statistically equivalent to azoxytrobin against L. nodorum and P. oryzae. Activity against the basidiomycete pathogen U. maydis is a little weaker, although at least equivalent to fluxapyroxad. The challenges of discovering a novel broad spectrum fungicide are illustrated by the commercial fungicide data in Table 1 which indicate that, with the possible exception of azoxytrobin, most products display differing levels of antifungal activity among this panel of plant pathogens. UK-2A and fenpicoxamid both have very little activity versus the oomycete pathogen P. infestans, inhibiting growth by less than 20% at 2 mg L⁻¹, the highest concentration tested against this pathogen. The data in Table 1 indicate fenpicoxamid to be slightly less active than UK-2A, ranging from 1.7× to 15× less active against P. oryzae and Z. tritici, respectively. Nonetheless, with the exception of U. maydis, inherent antifungal activity of fenpicoxamid remains very comparable to that of the commercial standards under the assay conditions employed. Of the many UK-2A derivatives prepared as part of the optimization process, the methyl ether (Fig. 1, R₁ = CH₃), also included in Table 1, is considered one of the more metabolically stable of the analogs. The EC50 values for this compound are considerably increased compared to both fenpicoxamid (ranging from 40- to 230-fold) and UK-2A (ranging from 270- to 1060-fold), implying less facile cleavage of the methyl ether compared to the isobutyryloxymethyl ether of fenpicoxamid.

3.1.2 Metabolism

Short term metabolism of fenpicoxamid was monitored both in Z. tritici (isolate ATCC 26518) cultures and wheat cell suspensions. The results of radio-high-performance liquid chromatography and mass spectral analysis of extracts of 48 h cultures of Z. tritici and 7-day-old wheat cell suspensions obtained after 3 h incubation with 14C-labeled fenpicoxamid are presented in Fig. 3. These data show that within 3 h of incubation fenpicoxamid (retention time, 11.4 min; [M+H]⁺, m/z 617.257) is almost completely converted to UK-2A (retention time, 9.4 min; [M+H]⁺, m/z 517.205) in both Z. tritici cultures and wheat cell suspensions.

Table 1. In vitro fungal growth inhibition by fenpicoxamid in comparison with commercial fungicides

| Compound            | L. nodorum (EC50 mg L⁻¹) | P. oryzae (EC50 mg L⁻¹) | Z. tritici (EC50 mg L⁻¹) | U. maydis (EC50 mg L⁻¹) | P. infestans (EC50 mg L⁻¹) |
|---------------------|--------------------------|-------------------------|-------------------------|-------------------------|---------------------------|
| Fenpicoxamid        | 0.082 (0.059, 0.115)      | 0.012 (0.009, 0.017)    | 0.051 (0.048, 0.054)    | 0.24 (0.15, 0.39)       | >2                        |
| UK-2A               | 0.013 (0.009, 0.019)      | 0.007 (0.005, 0.01)     | 0.0033 (0.003, 0.0036)  | 0.024 (0.013, 0.044)    | >2                        |
| UK-2A methyl ether  | 3.52 (1.60, 7.73)         | 2.77 (1.21, 6.36)       | 3.51 (1.59, 7.76)       | >18                     | nd                        |
| Azoxytrobin         | 0.02 (0.015, 0.027)       | 0.006 (0.005, 0.008)    | 0.0058 (0.0054, 0.0062) | 0.006 (0.0047, 0.0078)  | nd                        |
| Tebuconazole        | 0.06 (0.045, 0.079)       | 0.17 (0.12, 0.22)       | 0.077 (0.058, 0.103)    | 0.0035 (0.0029, 0.0042) | nd                        |
| Epoxiconazole       | 0.05 (0.04, 0.06)         | 0.41 (0.29, 0.58)       | 0.017 (0.016, 0.019)    | 0.008 (0.005, 0.013)    | nd                        |
| Fluxapyroxad        | >2                       | 0.79 (0.61, 1.03)       | 0.028 (0.026, 0.029)    | 0.025 (0.022, 0.028)    | nd                        |
| Isopyrazam          | >2                       | 0.016 (0.014, 0.019)    | 0.018 (0.011, 0.031)    | 0.002 (0.001, 0.005)    | nd                        |

nd, not tested.

3.2 Greenhouse testing

3.2.1 Disease spectrum

The ability of fenpicoxamid to provide strong control of both Z. tritici (isolate ATCC 26518) and P. triticina, two of the driver pathogens within the wheat market, is illustrated in Table 2 which shows EC80 values generated in low spray volume greenhouse tests conducted at both protectant and curative inoculation timings. Against Z. tritici, disease control is statistically equivalent to the SDHI fungicide fluxapyroxad. While epoxiconazole is some 10-fold and 4-fold more active as curative and protectant Z. tritici treatments, respectively, isolate ATCC 26518 retains very high sensitivity to both azole and strobilurin fungicide classes, a situation that no longer prevails in European wheat growing geographies. In these greenhouse tests against P. triticina, fenpicoxamid is some 2.5-fold more active than both fluxapyroxad and epoxiconazole as a protectant treatment and statistically equivalent to fluxapyroxad as a curative fungicide. Both fenpicoxamid and fluxapyroxad are ca 10-fold less efficacious than epoxiconazole as curative rust fungicides, which remains a strength of epoxiconazole.

Data capturing the activity of fenpicoxamid across a panel of seven additional plant pathogens are presented in Table 3. The values shown are EC80 values obtained in high spray volume tests sprayed 24 h prior to inoculation with spores of the respective pathogens. Comparison with the data generated for commercial fungicides epoxiconazole and fluxapyroxad suggests potential for additional utility for fenpicoxamid against diseases caused by C. lagenarium, P. oryzae, R. secalis and C. beticola. On the other hand, activity against V. inaequalis and A. solani is weak. Additional testing (data not shown) indicated that fenpicoxamid is only weakly active against Phakopsora pachyrhizi (Asian soybean rust) and powdery mildews and has little or no activity against the oomycete pathogens P. infestans (potato late blight) and Plasmopara viticola (grape downy mildew).

3.2.2 Comparative greenhouse activity of fenpicoxamid and UK-2A

Data presented in Table 4 reveal substantial differences between fenpicoxamid and UK-2A in their ability to control disease caused...
Figure 3. Conversion of $^{14}$C-fenpicoxamid to $^{14}$C-UK-2A in Z. tritici cultures and wheat cell suspensions. (Mass spectra of $^{14}$C-fenpicoxamid ([M+H]$^+$, m/z 617.257) and $^{14}$C-UK-2A ([M+H]$^+$, m/z 517.205) are shown in the insets.)

Table 2. Comparative activity of fenpicoxamid, fluxapyroxad and epoxiconazole versus Z. tritici and P. triticina in low volume greenhouse tests

|                | P. tritici 3-DC | P. triticina 1-DP | Z. tritici 3-DC | Z. tritici 1-DP |
|----------------|-----------------|-------------------|-----------------|-----------------|
| Fenpicoxamid   | 5.2 (3.7, 6.7)  | 2.7 (1.9, 3.4)    | 5.6 (3.2, 7.9)  | 6.8 (4.2, 9.5)  |
| Fluxapyroxad    | 7.8 (4.1, 11.5) | 6.7 (5.9, 7.6)    | 4.3 (2.5, 5.9)  | 7.4 (6.6, 8.3)  |
| Epoxiconazole   | 0.61 (0.59, 0.64)| 6.8 (4.5, 9.0)    | 0.47 (0.44, 0.50)| 1.8 (1.6, 2.0)  |

Values are EC$_{50}$ (g ha$^{-1}$); statistical analysis utilized the four-parameter Weibull model contained within the JMP Pro 11 software, the resulting 95% confidence limits being shown in parenthesis. Fluxapyroxad and epoxiconazole were applied as their commercial formulations Imtrex™ and Ignite™, respectively, and fenpicoxamid as an early stage EC formulation. 3-DC, plants sprayed 3 days post inoculation; 1-DP, plants sprayed 1 day prior to inoculation.

by both Z. tritici and P. triticina in high spray volume greenhouse tests. EC$_{50}$ values for fenpicoxamid were from 12- to 63-fold lower than those for UK-2A, depending on pathogen and inoculation timing (Table 4). These data show that although derivatization of the picolinamide OH group of UK-2A is not necessary for strong antifungal activity in growth inhibition assays (Table 1) it is a key requirement for realization of the full in planta disease control potential of this novel chemistry, including the excellent field performance delivered by fenpicoxamid against Z. tritici discussed below.

3.2.3 Cross-resistance studies
The ability of fenpicoxamid to provide strong control of isolates of Z. tritici resistant to strobilurin and azole fungicides is demonstrated by the data obtained in greenhouse tests (Table 5) made using four currently relevant isolates (UK-4, UK-7, UK-12 and UK-13) obtained from infected wheat leaf samples collected from trial sites in the UK. All four isolates are characterized as being resistant to both strobilurin and azole fungicide classes, sequence analysis confirming the presence of the G143A site mutation conferring strobilurin resistance in each case. Regarding azole resistance, the isolates carried the following mutations in the CYP51 gene encoding the sterol C-14 demethylase target site: UK-4, L50S, D134G, V136A, I381V and Y461H; UK-7, L50S, S188N, A379G, I381V, Y459/G460 deletion and N513K; UK-12, L50S, S188N, I381V, Y459/G460 deletion and N513K; UK-13, L50S, V136C, S188N, A379G, I381V, Y459/G460 deletion, N513K and S524T. Additionally, isolate UK-12 carries the 120 bp CYP51 promoter insert$^{13}$ as well as the 519 bp MgMFS1 promoter insert$^{23}$ that are linked with
overexpression of the respective genes. The 519 bp MgMFS1 promoter insert is also present in isolate UK-13.

The data presented in Table 5 are EC_{50} values generated from dose–response greenhouse tests conducted at low spray volumes and both 1 day protectant and 3 day curative inoculation timings. Representative strebolorin (pyraclostrobin), azole (epoxiconazole) and SDHi (fluxapyroxad) fungicides were included for comparison, as was ‘wild type’ Z. tritici isolate ATCC 26518, highly sensitive to all four chemistries. The data in Table 5 clearly show that the only isolate effectively controlled by the strobilurin and azole fungicides is ATCC 26518 whereas fenpicoxamid, although, in the majority of cases, EC_{80} values are statistically lower for fenpicoxamid than for fluxapyroxad. These data indicate that fenpicoxamid provides cereal growers with an important new tool for management of Z. tritici resistance. The lower sensitivity of UK-13 to both fluxapyroxad and fenpicoxamid could be related to overexpression of the non-specific efflux pump MgMFS1, mediated by the 519 bp promoter insert present in this isolate (B. Fraaije, personal communication). However, isolate UK-12 also contains the 519 bp MgMFS1 promoter insert but, nonetheless, is well controlled by both fenpicoxamid and fluxapyroxad (Table 5). The reason(s) for the difference in response between these two isolates is currently not clear and may warrant further investigation.

Table 3. Activity of fenpicoxamid against a broader spectrum of diseases in 1-day protectant high volume whole plant assays

| Pathogen (disease)          | EC_{80} values (mg L^{-1}) (95% confidence limits) | EC_{50} ratio UK-2A/fenpicoxamid |
|----------------------------|--------------------------------------------------|----------------------------------|
|                            | Fenpicoxamid                                     | Epoxiconazole                    | Fluxapyroxad                      |
| A. solani (tomato early blight) | >100                                             | 12.72 (11.07, 14.62)             | 2.53 (1.13, 5.64)                |
| C. beticola (sugar beet leaf spot) | 10.78a                                           | 2.88 (1.47, 5.62)                | 1.77 (0.56, 5.59)                |
| C. lagenarium (cucumber anthracnose) | 3.29 (1.06, 10.22)                              | 3.91 (2.55, 5.99)                | >100                             |
| L. nodorum (glume blotch) | 0.12 (0.03, 0.5)                                 | 0.31 (0.14, 0.67)                | 58.65 (27.99, 122.9)             |
| P. oryzae (rice blast)        | 5.58 (2.24, 13.91)                               | 7.54 (5.24, 10.85)               | >100                             |
| R. secale (barley scald)       | 7.54 (2.59, 22.0)                                | 1.96 (1.60, 2.39)                | 1.56 (1.40, 1.74)                |
| V. inaequalis (apple scab)     | 81.79 (28.73, 232.8)                             | 15.8 (3.94, 63.34)               | 3.87 (0.63, 23.58)               |

Statistical analysis was completed using the two-parameter logistic model contained within the R (RProj) statistical software, the resulting 95% confidence limits being shown in parenthesis. All three fungicides were formulated in a generic laboratory formulation consisting of 10% aqueous acetone supplemented with 100 mg L^{-1} Triton X-100 and applied at high spray volumes.

Table 4. Relative efficacy of fenpicoxamid and UK-2A versus Z. tritici and P. triticina in greenhouse tests

| Pathogen       | Timing | Compound | EC_{50} value (mg L^{-1}) (95% confidence limits) | EC_{50} ratio UK-2A/fenpicoxamid |
|----------------|--------|----------|--------------------------------------------------|----------------------------------|
| Z. tritici     | 1-DP   | UK-2A    | 6.04 (5.06, 7.02)                                 | 12.33                            |
| Z. tritici     | 1-DP   | Fenpicoxamid | 0.49 (0.40, 0.58)                               |                                   |
| Z. tritici     | 3-DC   | UK-2A    | 83.7 (nd)                                        | 63.41                            |
| Z. tritici     | 3-DC   | Fenpicoxamid | 1.32 (1.11, 1.55)                               |                                   |
| P. triticina   | 1-DP   | UK-2A    | 3.71 (3.38, 4.04)                                | 23.19                            |
| P. triticina   | 1-DP   | Fenpicoxamid | 0.16 (0.15, 0.17)                               |                                   |
| P. triticina   | 3-DC   | UK-2A    | 36.05 (23.98, 48.12)                             | 43.96                            |
| P. triticina   | 3-DC   | Fenpicoxamid | 0.82 (0.64, 1.00)                               |                                   |

Statistical analysis was completed using the four-parameter Weibull model contained within the JMP Pro 11 software, the resulting 95% confidence limits being shown in parenthesis. 1-DP and 3-DC, 1 day protectant and 3 day curative application timings respectively. Compounds were formulated in 10% aqueous acetone /100 mg L^{-1} Triton X-100 and applied at high spray volumes.
Table 5. Comparative control of fungicide resistant isolates of *Z. tritici* by fenpicoxamid and current cereal fungicides in low volume greenhouse tests

| Fungicide       | Timing | ATCC 26518 | UK-4   | UK-7   | UK-12  | UK-13  |
|-----------------|--------|------------|--------|--------|--------|--------|
| Fenpicoxamid    | 1-DP   | 1.8 (1.6, 1.9) | 1.8 (1.6, 2.1) | 1.3 (1.1, 1.5) | 1.2 (0.91, 1.5) | 6.2 (1.6, 10.8) |
|                 | 3-DC   | 2.2 (1.8, 2.5) | 1.6 (1.4, 1.7) | 1.7 (1.6, 1.8) | 1.6 (1.3, 1.9) | 17.2 (12.3, 22.1) |
| Fluxapyroxad    | 1-DP   | 6.7 (6.5, 6.9) | 6.8 (6.5, 7.0) | 6.9 (5.5, 8.4) | 5.8 (4.8, 6.7) | 27 (26.4, 27.5) |
|                 | 3-DC   | 4.6 (3.9, 5.4) | 4.3 (2.1, 6.5) | 3.2 (1.1, 5.3) | 7.3 (0.72, 13.8) | 27.9 (17.8, 38.1) |
| Epoxiconazole   | 1-DP   | 4.2 (1.0, 7.3) | 98.3b   | >100    | >100   | >100   |
|                 | 3-DC   | 1.1 (0.96, 1.3) | 8.72b   | 17.7 (5.5, 30) | >100   | >100   |
| Pyraclostrobin  | 1-DP   | 3.8 (1.5, 6.2) | >100    | >100    | >100   | >100   |
|                 | 3-DC   | 1.7 (1.2, 2.2) | >100    | >100    | >100   | >100   |

Values are EC₈₀ (g. as ha⁻¹); statistical analysis utilized the four-parameter Weibull model contained within the JMP Pro 11 software, the resulting 95% confidence limits being shown in parenthesis. Fluxapyroxad, epoxiconazole and pyraclostrobin were applied as their commercial formulations Imtrex™, Ignite™ and Headline™, respectively, and fenpicoxamid as an early stage EC formulation.

*UK-4, UK-7, UK-12 and UK-13 are recent field isolates of *Z. tritici* from the UK, whereas ATCC 26518 is a USA-derived isolate highly sensitive to both strobilurin and triazole fungicides.*

*Confidence limits not determined.*

Figure 4. Leaf bioassays showing redistribution of fenpicoxamid from mid-leaf points of application. (Fenpicoxamid was applied at 1 μg per leaf 24 h prior to inoculation with *P. triticina.*

Based on assessments made 6–9 weeks after application when differences between fenpicoxamid, prothioconazole (used as the reference commercial product) and the untreated controls were most obvious. When percentage control means from all trials were analyzed for variance (restricted maximum likelihood estimation method), the results clearly indicated treatment to be a significant component (F₁,₉ = 31.61, P = 0.0003). Subsequent comparison of the least square means obtained for fenpicoxamid and prothioconazole across all 10 trials (Table 6) using Tukey’s HSD test showed that there are significant differences between the two treatments. These data clearly illustrate the superior residuality of fenpicoxamid, applied between winter wheat growth stages BBCH 32 and 39 at the proposed label rate of 100 g ha⁻¹, for control of *Z. tritici* infections, particularly considering the 2–4 week incubation period until first symptoms appear on leaves. Thus the efficacy pattern found in these 10 field trials, conducted across European geographies, showed that fenpicoxamid provided excellent *Z. tritici* control over a 2 month period from a single application. Additionally, no crop injury or negative impact on yield or components of yield have been observed when fenpicoxamid formulations have been tested in over 400 field trials in Europe on multiple wheat varieties under a range of growing conditions.
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The data presented above showing strong control of Fenpicoxamid, as evidenced in both greenhouse testing and field (Table 6) efficacy delivered by Fenpicoxamid.

Table 6. Mean efficacy of fenpicoxamid against Z. tritici determined across 10 field trials

| Percent infection on untreated controls (leaves 1–3) | Percent control of Z. tritici$^a$ |
|-----------------------------------------------------|---------------------------------|
| Fenpicoxamid, 100 g as ha$^{-1}$ | 82.1$^{b}$ (65.2, 95.3) |
| Prothioconazole, 198 g as ha$^{-1}$ | 68.2$^{b}$ (44.5, 81.7) |
| 47.2 (16.2, 76.7) | 54.2 (37.5, 65.9) |

Prothioconazole was applied as Proline 275™ (Bayer CropScience) and fenpicoxamid as an early stage EC formulation.

$^a$ Percent control data are least square mean values across leaves 1, 2 and 3 for all 10 trials.

$^b$ Means are significantly different based on Tukey’s HSD comparison. Numbers in parenthesis represent the range of means (minimum, maximum) determined for individual trials.

3.4 Comparative surface stability studies of fenpicoxamid and UK-2A

Despite the strong antifungal activity of UK-2A itself evident in Table 1, particularly against Z. tritici, the ability of the natural product to control this and other cereal diseases in greenhouse tests (Table 4) is weaker than might be expected. Physical properties were considered to be one possible factor involved in limiting its in planta activity translation, and protection of the ionizable picolinamide OH group (pK$_a$ 8.48) through derivatization drove the synthetic effort that led to identification of fenpicoxamid as a development candidate. Of greater significance, however, is the surface instability of UK-2A when applied not only to wheat leaves but also to abiotic filter paper discs. The data in Fig. 5(A) indicate that the recovery of UK-2A and fenpicoxamid from wheat leaf surfaces within 24 h of application of 25 ppm spray solutions is, respectively, 9.3% and 103.5%, the >100% figure representing the variation in the amounts deposited on leaf surfaces by high spray volume applications. Further studies (Fig. 5(B)) indicated that extensive loss of UK-2A also occurred when it was applied to inert filter paper discs regardless of whether they were maintained in the dark or under ambient light conditions, recovery being only some 30% after 24 h. Exposure to UV light for 24 h resulted in complete loss of UK-2A. Under the same conditions fenpicoxamid is very stable, with only 20% loss occurring during the UV exposure conditions. Chromatographic analysis of material recovered from the filter paper discs indicated formation of several degradates that probably result from oxidative processes. The unequivocal identification of these continues to be pursued via a separate research effort outside the scope of the present paper. Clearly the picolinamide hydroxyl group plays a role in predisposing UK-2A to surface and UV-mediated degradation phenomena, since its derivatization to the isobutyryloxymethyl ether addressed the issue very effectively. These differences in surface stability between UK-2A and fenpicoxamid present a highly plausible explanation not only for the weaker greenhouse activity of UK-2A (Table 4) but also for the compelling greenhouse (Tables 2–5) and field (Table 6) efficacy delivered by fenpicoxamid.

3.5 Scanning electron microscopy

The data presented above showing strong control of Z. tritici by fenpicoxamid applied to host leaves several days post inoculation clearly indicate its ability to disrupt hyphal growth and development post spore germination, either prior to or post stomatal penetration, or both. Confocal microscopy was used to examine the impact of a 1 day protectant application of fenpicoxamid at 6.25 ppm on the germination and growth of a GFP-expressing Z. tritici strain on leaf surfaces of wheat seedlings 72 h after inoculation (Fig. 6). In the case of untreated controls, at low magnification, hyphae can be seen to have developed and colonized the leaf surface quite extensively, in some cases extending several hundred microns (Fig. 6(A)). On fenpicoxamid treated leaves, on the other hand, most of the spores appear unable to germinate within 72 h of inoculation (Fig. 6(B)). At higher magnification, using ESEM, some of the spores on the surface of treated leaves can be seen to have germinated, but germ tube length is dramatically reduced (Fig. 6(D)) compared to the untreated samples (Fig. 6(C)). Given that fenpicoxamid effectively inhibits Z. tritici when applied to leaves post infection (curative timing, Tables 2, 4 and 5), these data probably reflect an ability of fenpicoxamid to inhibit hyphal growth of Z. tritici on host leaf surfaces also.

4 CONCLUSION

Fenpicoxamid is a new fungicide active ingredient which was identified and developed by semi-synthetic modification of the naturally occurring antifungal compound UK-2A. It is strongly active against Z. tritici, as evidenced in both greenhouse testing and extensive field trials in Europe, and thus has an excellent fit for
use in the cereals markets. Unlike UK-2A, fenpicoxamid has excellent stability on leaf surfaces such that residual protection is a key strength, although it is also efficacious against latent infections present in leaves at the time of application. This chemistry delivers a novel mode of action for the cereals fungicide market, involving inhibition of the mitochondrial respiratory bc1 complex at the Q_i binding site, which is distinct from the Q_o site to which the strobilurins bind. Fenpicoxamid therefore provides excellent control of fungal isolates resistant to strobilurins and also to other classes of chemistry, such as azoles, thus offering the grower a powerful new tool for resistance management. The supporting information shows that, while toxicity to non-target organisms in the environment is generally very low, fenpicoxamid has significant toxicity to some aquatic organisms such as fish, similar to other fungicides that inhibit respiration. However, extensive risk mitigation measures are available in Europe allowing environmentally safe usage of fenpicoxamid.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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