ICCR ROßDORF

Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram

Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (A23793B) - *Salmonella Typhimurium* and *Escherichia Coli* Reverse Mutation Assay

Final Report

TEST GUIDELINE(S): OECD 471 (2020)

AUTHOR(S): Dr. Steffi Chang

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PERFORMING LABORATORY: ICCR-Roßdorf GmbH
In den Leppsteinswiesen 19
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LABORATORY PROJECT ID: Report Number: 2190200
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SPONSOR(S): Syngenta Ltd.
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Bracknell, Berkshire RG42 6EY, United Kingdom

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This study performed in the test facility of ICCR-Roßdorf GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), in its currently valid version

OECD Principles of Good Laboratory Practice, (as revised in 1997), ENV/MC/CHEM(98)17

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There were no circumstances that may have affected the quality or integrity of the study.

Dr. Steffi Chang
Study Director Bacterial Systems

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Date: 05 April 2022

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QUALITY ASSURANCE STATEMENT

ICCR Study Number: 2190200
Test substance: Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (A23793B)
Study director: Dr. Steffi Chang
Study Title: Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (A23793B): *Salmonella Typhimurium* and *Escherichia Coli* Reverse Mutation Assay

Study based activities at the Test Facility ICCR-Roßdorf GmbH were audited and inspected. The details of these audits and inspections are given below.

Type of Inspection	Date(s) of Inspection	Date Reporting to Study Director, Test Facility Management
Study Plan Verification	09 December 2021	09 December 2021
Process – based Assessment of Response	13 January 2022	13 January 2022
Report Audit	16 March 2022	16 March 2022

General facilities and activities where this study was conducted were inspected on an annual basis and results are reported to the relevant responsible person and Management.

The statement is to confirm, that this report reflects the raw data.

S. Ebert

Sabine Ebert
Quality Assurance Auditor
ICCR-Roßdorf GmbH

01 April 2022
Date

PROJECT STAFF SIGNATURE

Study Director — Dr. Steffi Chang

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Date: 05 April 2022

GENERAL INFORMATION

Contributors
The following contributed to this report in the capacities indicated:

Name	Title
Dr. Steffi Chang	Study Director
Dr. Markus Schulz	Test Facility Management
Frauke Hermann	Head of Quality Assurance Unit
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Study Dates
Study initiation date: 10 December 2021
Experimental start date: 14 December 2021
Experimental completion date: 19 January 2022

Deviations from the Guidelines
None

Retention of Samples
None

Performing Laboratory Test Substance Reference Number
S 2194111

Other
ICCR-Roßdorf GmbH will archive:
Records and documentation relating to this study will be maintained in the archives of ICCR-Roßdorf GmbH for a period of 4 years from the date on which the Study Director signs the final report. This will include electronic and paper raw data, and report that support the reconstruction of the study.

At termination of the aforementioned period, the records and documentation will be transferred to the GLP compliant Archive of Rhenus Archiv Services GmbH, Frankfurt am Main for further archiving up to a total archiving period of 15 years.

A sample of the test substance will not be archived.

ICCR Roßdorf GmbH will retain in its archive a copy of the study plan and final report, and any amendments indefinitely.

Deviations from the study plan
There were no deviations (unplanned changes) from the study plan.

Distribution of the report

Sponsor	2 electronic copies (1 pdf-file, 1 word-file)
Study Director	1 (original)

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

This study was performed to investigate the potential of difenoconazole/fludioxonil/metalaxyl-M/cyclobutrifluram FS (A23793B) to induce gene mutations in the plate incorporation test (Experiment I) and the pre-incubation test (Experiment II) using the *Salmonella typhimurium (S. typhimurium)* strains TA1535, TA1537, TA98, and TA100, and the *Escherichia coli (E. coli)* strains WP2 *uvrA* (pKM101) and WP2 (pKM101).

1.2 Results

The plates incubated with the test substance showed normal background growth up to the maximal concentration of 5000 µg/plate with and without S9 mix in all strains used.

A single minor cytotoxic effect, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in Experiment II in strain WP2 (pKM101) without metabolic activation.

No relevant increase in revertant colony numbers of any of the six tester strains was observed following treatment with difenoconazole/fludioxonil/metalaxyl-M/cyclobutrifluram FS (A23793B) at any concentration, neither in the presence nor absence of metabolic activation (S9 mix). There was also no observed tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls, which showed a distinct increase of induced revertant colonies consistent with the laboratory's historical control data demonstrated the sensitivity of the test system and the efficacy of the S9 mix. Each batch of S9 was also tested with 2 pro-mutagens, benzo(a)pyrene and 2-aminoanthracene.

1.3 Conclusion

In conclusion, it can be stated that during the described mutagenicity tests and under the experimental conditions reported, difenoconazole/fludioxonil/metalaxyl-M/cyclobutrifluram FS (A23793B) did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, difenoconazole/fludioxonil/metalaxyl-M/cyclobutrifluram FS (A23793B) is considered to be negative (i.e. non-mutagenic) in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

2.0 INTRODUCTION

2.1 Purpose

These experiments were performed to assess the potential of the test substance to induce gene mutations by means of the *S. typhimurium* and *E. coli* reverse mutation assay. Experiment I was performed as a plate incorporation assay. Since a negative result was obtained in this experiment, Experiment II was performed as a pre-incubation assay.

The most widely used assays for detecting gene mutations are those using bacteria (1). They are relatively simple and rapid to perform, and give reliable data on the ability of an agent to interact with DNA and produce mutations.

Reverse mutation assays determine the frequency with which an agent reverses or suppresses the effect of the forward mutation. The genetic target presented to an agent is therefore small, specific and selective. Several bacterial strains, or a single strain with multiple markers are necessary to assure reliable detection of mutagens that may be specific to one tester strain or locus. The reversion of bacteria from growth-dependence on a particular amino acid to growth in the absence of that amino acid (reversion from auxotrophy to prototrophy) is the most widely used marker.

The S. *typhimurium* histidine (his) and the *E. coli* tryptophan (trp) reversion system measures $his^- \rightarrow his^+$ and $trp^- \rightarrow trp^+$ reversions, respectively. The *S. typhimurium* and *E. coli* strains are constructed to differentiate between base pair (TA1535, TA100, WP2 *uvrA* (pKM101), and WP2 (pKM101)) and frameshift (TA1537, TA98) mutations.

According to the direct plate incorporation and pre-incubation method the bacteria are exposed to the test substance with and without metabolic activation and plated on selective medium. After a suitable period of incubation, revertant colonies are counted.

To establish a concentration response effect at least six concentrations with adequately spaced intervals were tested. The maximum concentration was 5000 µg/plate.

To validate the test, reference mutagens were tested in parallel to the test substance.

2.2 Test Guideline(s)

This study followed the procedures indicated by the following internationally accepted guideline and recommendations:

"Ninth Addendum to OECD Guidelines for Testing of Chemicals", Section 4, No. 471: "Bacterial Reverse Mutation Test", corrected June 26, 2020

3.0 MATERIALS AND METHODS

3.1 Test Substance

Information as provided by the Sponsor.

Identification:	Difenoconazole/Fludioxonil/Metalaxyl-M/ Cyclobutrifluram FS (A23793B)
Batch:	1200767
Content of	
Difenoconazole:	5.45% w/w corresponding to 64.0 g/L
Fludioxonil:	4.37% w/w corresponding to 51.3 g/L
Metalaxyl-M:	4.31% w/w corresponding to 50.6 g/L
Cyclobutrifluram:	21.0% w/w corresponding to 247 g/L
Appearance:	Red, liquid
Recertification Date:	31 August 2024
Storage Conditions:	At room temperature
Stability in Solvent:	Not indicated by the Sponsor

The test substance concentrations were neither adjusted for the content of difenoconazole, nor fludioxonil or metalaxyl-M or cyclobutrifluram.

On the day of the experiment (immediately before use), the test substance was dissolved in dimethylsulfoxide (DMSO, purity > 99%). The solvent was chosen as the best suitable solvent compared to water and ethanol, according to its solubilisation properties and its relative non-toxicity to the bacteria (2).

All formulations were prepared freshly before treatment and used within two hours of preparation. The formulation was assumed to be stable for this period unless specified otherwise by the Sponsor.

3.2 Controls

3.2.1 Negative controls

Concurrent untreated and solvent controls were performed.

3.2.2 Positive control substances

Without metabolic activation

Strains:	TA1535, TA100
Name:	Sodium azide, (NaN_3)
Supplier:	Sigma-Aldrich, 82024 Taufkirchen, Germany
Batch No.:	STBJ7813
Purity:	≥ 99%
Dissolved in:	Deionised water
Concentration:	10 µg/plate

Strains:	TA1537, TA98
Name:	4-nitro-o-phenylene-diamine, (4-NOPD)
Supplier:	Sigma-Aldrich, 82024 Taufkirchen, Germany
Batch No.:	MKBM 5257V
Purity:	≥ 98%
Dissolved in:	DMSO (purity >99 %, Fisher Leics LE11 5RG, United Kingdom)
Concentration:	10 µg/plate in strain TA 98, 50 µg/plate in strain TA 1537

Strains:	WP2 *uvrA* (pKM101), WP2 (pKM101)
Name:	Methyl methane sulfonate, (MMS)
Supplier:	Sigma-Aldrich, 82024 Taufkirchen, Germany
Batch No.:	MKCL 6261
Purity:	≥ 99%
Dissolved in:	Deionised water
Concentration:	2.0 µL/plate

With metabolic activation

Strains:	TA1535, TA1537, TA98, TA100, WP2 *uvrA* (pKM101), WP2 (pKM 101)
Name:	2-aminoanthracene, (2-AA)
Supplier:	Sigma-Aldrich, 82024 Taufkirchen, Germany
Batch No.:	STBG 0630V
Purity:	≥ 96%
Dissolved in:	DMSO (purity > 99 %, Fisher Leics LE11 5RG, United Kingdom)
Concentration:	2.5 µg/plate (TA1535, TA1537, TA98, TA100), 10 µg/plate (WP2 *uvrA* (pKM101), WP2 (pKM101))

The stability of the positive control substances in solution is unknown but a mutagenic response in the expected range is sufficient evidence of biological activity.

3.3 Experimental Design

3.3.1 Characterisation of the *Salmonella typhimurium* and *E. coli* strains

The histidine dependent strains are derived from *S. typhimurium* strain LT2 through mutations in the histidine locus. Additionally, due to the "deep rough" (*rfa*$^-$) mutation they possess a faulty lipopolysaccharide envelope which enables substances to penetrate the cell wall more easily. A further mutation causes a reduction in the activity of an excision repair system. The last alteration includes mutational processes in the nitrate reductase and biotin genes produced in a UV-sensitive area of the gene named *uvrB*$^-$. In the strains TA98 and TA100 the R-factor plasmid pKM101 carries the ampicillin resistance marker (3).

Strain WP2 (4) and its derivatives all carry the same defect in one of the genes for tryptophan biosynthesis. Tryptophan-independent (*Trp*$^+$) mutants (revertants) can arise either by a base change at the site of the original alteration or by a base change elsewhere in the chromosome so that the original defect is suppressed. This second possibility can occur in several different ways so that the system seems capable of detecting all types of mutagen which substitute one base for another. Additionally, the *uvrA* derivative is deficient in the DNA repair process (excisable repair damage). Such a repair-deficient strain may be more readily mutated by agents. The *E. coli* strains WP2 *uvrA* (pKM101) and WP2 (pKM101) are constructed by introduction of the R-factor plasmid pKM101.

When summarized, the mutations of the *S. typhimurium* and *E. coli* strains used in this study can be described as follows:

Strains	**Genotype**	**Type of mutations indicated**
	Salmonella typhimurium	
TA1537	*his* C 3076; *rfa*$^-$; *uvrB*$^-$	frame shift mutations
TA98	*his* D 3052; *rfa*$^-$; *uvrB*$^-$; R-factor	" "
TA1535	*his* G 46; *rfa*$^-$; *uvrB*$^-$	base-pair substitutions
TA100	*his* G 46; *rfa*$^-$; *uvrB*$^-$; R-factor	" "
	Escherichia coli	
WP2 *uvrA* (pKM101)	*trp* E 56 *uvrA*$^-$; R-factor	base-pair substitutions and others
WP2 (pKM101)	*trp* E 56; R-factor	" "

Regular checking of the properties of the *S.typhimurium* and *E. coli* strains regarding the membrane permeability and ampicillin resistance; UV sensitivity, and amino acid requirement as well as normal spontaneous mutation rates is performed by ICCR-Roßdorf GmbH according to Ames *et al.* (5), Maron and Ames (3), and Mortelmans and Riccio (7). In this way it is ensured that the experimental conditions set down by Ames are fulfilled.

The bacterial strains TA1535, TA1537, TA98, TA100, WP2 *uvrA* (pKM101), and WP2 (pKM101) were obtained from Trinova Biochem GmbH (35394 Gießen, Germany).

3.3.2 Storage

The strain cultures were stored as stock cultures in ampoules with nutrient broth + 5 % DMSO (Fisher Leics, LE11 5RG, United Kingdom) in liquid nitrogen.

3.3.3 Precultures

The thawed bacterial suspension was transferred into 250 mL Erlenmeyer flasks containing nutrient medium (50 mL). A solution of ampicillin (50 µL, 25 µg/mL) was added to the strains TA98, TA100, WP2 *uvrA* (pKM101), and WP2 (pKM101). This nutrient medium contains per liter:

8 g Nutrient Broth (MERCK, 64293 Darmstadt, Germany)
5 g NaCl (MERCK, 64293 Darmstadt, Germany)

The bacterial cultures were incubated in a shaking water bath for 4 hours at 37° C. The optical density of the bacteria was determined by absorption measurement and the obtained values indicated that the bacteria were harvested at the late exponential or early stationary phase (10^8-10^9 cells/mL).

3.3.4 Selective agar

Plates with selective agar (without Histidine/Tryptophan) were used.

3.3.5 Overlay agar

The overlay agar contained per litre:

for *Salmonella* strains:	for *Escherichia coli* strains:
7.0 g Agar Agar*	7.0 g Agar Agar*
6.0 g NaCl*	6.0 g NaCl*
10.5 mg L-Histidine×HCl×H_2O*	10.2 mg Tryptophan*
12.2 mg Biotin*	

* (MERCK, 64293 Darmstadt, Germany)

Sterilisations were performed at 121° C in an autoclave.

3.4 Mammalian Microsomal Fraction S9 Mix

The bacteria used in this assay do not possess the enzyme systems which, in mammals, are known to convert promutagens into active DNA damaging metabolites. In order to overcome this major drawback an exogenous metabolic system is added in the form of mammalian microsome enzyme activation mixture.

3.4.1 S9 (Preparation by ICCR-Roßdorf GmbH)

Phenobarbital/ß-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Wistar rats (RjHan:WI; weight approx. 220 – 320 g,

Janvier Labs, 53941 Saint-Berthevin Cedex, France) induced by peroral administration of 80 mg/kg b.w. phenobarbital (Sigma-Aldrich Chemie GmbH, 82024 Taufkirchen, Germany) and by peroral administrations of ß-naphthoflavone (Acros Organics, 2440 Geel, Belgium) each, on three consecutive days. The livers were prepared 24 hours after the last treatment. The S9 fractions were produced by dilution of the liver homogenate with a KCl solution (1+3 parts) followed by centrifugation at 9000 g. Aliquots of the supernatant were frozen and stored in ampoules at –80 °C. Small numbers of the ampoules can be kept at –20 °C for up to one week. Each batch of S9 mix is routinely tested with 2-aminoanthracene as well as benzo[a]pyrene (Appendix 3).

The protein concentration in the S9 preparation was 29.6 mg/mL (lot no. 080721D) in both experiments.

3.4.2 S9 mix

Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution. The amount of S9 supernatant was 10% v/v in the S9 mix. Cofactors were added to the S9 mix to reach the following concentrations in the S9 mix:

8 mM	$MgCl_2$
33 mM	KCl
5 mM	Glucose-6-phosphate
4 mM	NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.
During the experiment the S9 mix was stored in an ice bath. The S9 mix preparation was performed according to Ames *et al.*(5).

3.5 Pre-Experiment for Cytotoxicity

To evaluate the cytotoxicity of the test substance a pre-experiment was performed with all strains. Eight concentrations were tested for cytotoxicity and mutation induction each with three replicate plates. The experimental conditions in this pre-experiment are described in section 3.7 (plate incorporation test).

Cytotoxicity of the test substance results in a reduction in the number of spontaneous revertants (below a factor of 0.5) or a clearing of the bacterial background lawn.

The pre-experiment is reported as the Main Experiment I since the criteria mentioned in Section 3.8.2 Acceptability of the Assay were met.

3.6 Concentration Selection

In the pre-experiment the concentration range of the test substance was 3 - 5000 µg/plate. The pre-experiment is reported as Experiment I. Since no cytotoxic effects were observed 5000 µg/plate was chosen as the maximal concentration in Experiment II.

The concentration range included two logarithmic decades. The following concentrations were tested in Experiment II:

33; 100; 333; 1000; 2500; and 5000 µg/plate

3.7 Experimental Performance

For each strain and concentration including the controls, three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

100 µL	Test solution at each concentration, solvent (negative control) or reference mutagen solution (positive control),
500 µL	S9 mix (for test with metabolic activation) or S9 mix substitution buffer* (for test without metabolic activation),
100 µL	Bacteria suspension (cf. test system, pre-culture of the strains; OD = 0.9 - 1.2; wavelength = 500 nm; approx. $8x10^8$ cells/mL),
2000 µL	Overlay agar

For the pre-incubation method test solution (100 µL) (solvent or reference mutagen solution (positive control)), S9 mix / S9 mix substitution buffer* (500 µL) and bacteria suspension (100 µL) were mixed in a test tube and incubated at 37° C ± 1.5° C for 60 minutes. After pre-incubation overlay agar (2.0 mL, 45° C) was added to each tube. The mixture was poured on selective agar plates.

After solidification the plates were incubated upside down for 72 hours at 37° C ± 1.5° C in the dark, plates were then stored at 4° C until counted (6).

In parallel to each test a sterile control of the test substance was performed and documented in the raw data. Therefore, stock solution (100 µL) and S9 mix / S9 mix substitution buffer* (500 µL) were mixed with overlay agar (2.0 mL) and poured on minimal agar plates.

* Substitution buffer: 7 parts of the 100 mM sodium-ortho-phosphate-buffer pH 7.4 with 3 parts of KCl solution 0.15 M

3.8 Data Evaluation

3.8.1 Data recording

The colonies were counted using a Petri Viewer with the software program Ames Study Manager (see section 3.9, Major computerized systems). The evaluation unit was connected

to a PC with printer to print out the individual values, the means from the plates for each concentration together with standard deviations and enhancement factors as compared to the spontaneous reversion rates (see tables of results). The print outs are kept with the raw data. Due to precipitation of the test item some test groups were scored manually (as indicated on data tables).

3.8.2 Acceptability of the assay

The *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay is considered acceptable if it meets the following criteria:

- regular background growth in the negative and solvent control
- the spontaneous reversion rates in the negative and solvent control are in the range of the historical data
- the positive control substances should produce an increase above the threshold of twofold (strains TA 98, TA 100, WP2 uvrA (pKM101, and WP2 (pKM101))) or threefold (strains TA 1535 and TA 1537) the revertant colony count of the corresponding solvent control;
- a minimum of five analysable concentrations should be present with at least four showing no signs of toxic effects, evident as a reduction in the number of revertants below the indication factor of 0.5.

3.8.3 Evaluation of results

A test substance is considered as a mutagen if a biologically relevant increase in the number of revertants of twofold or above (strains TA 98, TA 100, WP2 uvrA (pKM101), and WP2 (pKM101)) or of threefold or above (strains TA 1535 and TA 1537) the spontaneous mutation rate of the corresponding solvent control is observed (1).

A concentration dependent increase is considered biologically relevant if the threshold is reached or exceeded at more than one concentration (6).

An increase of revertant colonies equal or above the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

A concentration dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls, such an increase is not considered biologically relevant.

3.8.4 Biometry

According to the OECD guideline 471, a statistical analysis of the data is not mandatory.

3.9 Major Computerized System

Petri Viewer Sorcerer Colony Counter 3.0 (Instem, Suffolk IP33 3TA, UK) with the software program Ames Study Manager (v1.24) and Ames Archive Manager (v1.01).

4.0 RESULTS AND DISCUSSION

The test substance, difenoconazole/fludioxonil/metalaxyl-M/cyclobutrifluram FS (A23793B), was assessed for its potential to induce gene mutations in the plate incorporation test (Experiment I) and the pre-incubation test (Experiment II) using *S. typhimurium* strains TA1535, TA1537, TA98, and TA100, and the *E. coli* strains WP2 (pKM101) and WP2 *uvrA* (pKM101).

In the pre-experiment the concentration range of the test substance was 3 - 5000 µg/plate. The pre-experiment is reported as Experiment I. Since no cytotoxic effects were observed 5000 µg/plate was chosen as the maximal concentration in Experiment II. This is the maximum concentration recommended in the OECD test guideline.

The assay was performed with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The concentration range included two logarithmic decades. The test substance was tested at the following concentrations:

Pre-Experiment/Experiment I:	3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment II:	33; 100; 333; 1000; 2500; and 5000 µg/plate

The test substance precipitated in the overlay agar in the test tubes from 2500 to 5000 µg/plate. Precipitation of the test item in the overlay agar on the incubated agar plates was observed at 5000 µg/plate.

The plates incubated with the test substance showed normal background growth up to the maximal concentration of 5000 µg/plate with and without S9 mix in all strains used.

A single minor cytotoxic effect, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in Experiment II in strain WP2 (pKM101) without metabolic activation at 5000 µg/plate. No cytotoxic effects occurred in Experiment I and the remaining test groups of Experiment II.

No substantial increase in revertant colony numbers in any of the six tester strains was observed following treatment with difenoconazole/fludioxonil/metalaxyl-M/cyclobutrifluram FS (A23793B) at any concentration, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

5.0 CONCLUSIONS

In conclusion, it can be stated that during the described mutagenicity tests and under the experimental conditions reported, difenoconazole/fludioxonil/metalaxyl-M/cyclobutrifluram

FS (A23793B) did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, difenoconazole/fludioxonil/metalaxyl-M/cyclobutrifluram FS (A23793B) is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

6.0 REFERENCES

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TABLES SECTION

TABLE 1 Summary of Results Pre-Experiment/Experiment I

Study Name: 2190200
Experiment: 2190200 VV Plate
Assay Conditions:

Study Code: ICCR 2190200
Date Plated: 14.12.2021
Date Counted: 17.12.2021

Metabolic Activation	Test Group	Concentration (per plate)	Revertant Colony Counts (Mean ±SD)					
			TA 1535	TA 1537	TA 98	TA 100	WP2 pKM101	WP2 uvrA pKM101
Without Activation	DMSO		11 ± 3	10 ± 4	33 ± 3	124 ± 15	294 ± 2	325 ± 11
	Untreated		13 ± 2	10 ± 1	31 ± 6	128 ± 11	322 ± 27	359 ± 3
	Difenoconazole/	3 µg	11 ± 1	12 ± 0	27 ± 9	124 ± 6	283 ± 28	388 ± 14
	Fludioxonil/	10 µg	15 ± 2	11 ± 3	33 ± 9	123 ± 17	261 ± 3	305 ± 18
	Metalaxyl-M/	33 µg	14 ± 3	9 ± 2	36 ± 4	125 ± 10	273 ± 15	296 ± 16
	Cyclobutrifluram	100 µg	11 ± 4	9 ± 3	26 ± 5	136 ± 9	240 ± 8	327 ± 39
	FS (A23793B)	333 µg	12 ± 4	12 ± 2	23 ± 7	124 ± 18	273 ± 10	326 ± 23
		1000 µg	10 ± 3	10 ± 3	30 ± 10	118 ± 16	229 ± 7	280 ± 32
		2500 µg	10 ± 3	6 ± 1	26 ± 8	130 ± 5	209 ± 22	258 ± 28
		5000 µg	9 ± 3 [P]	10 ± 1 [P]	23 ± 7 [P]	120 ± 10 [P]	163 ± 21 [P]	223 ± 21 [P]
	NaN3	10 µg	1175 ± 47			1673 ± 105		
	4-NOPD	10 µg			641 ± 15			
	4-NOPD	50 µg		109 ± 10				
	MMS	2.0 µL					2970 ± 146	2898 ± 168
With Activation	DMSO		10 ± 2	14 ± 3	45 ± 13	134 ± 12	286 ± 9	398 ± 31
	Untreated		14 ± 3	13 ± 3	46 ± 2	128 ± 14	340 ± 9	397 ± 29
	Difenoconazole/	3 µg	10 ± 3	12 ± 2	42 ± 12	104 ± 1	304 ± 6	469 ± 56
	Fludioxonil/	10 µg	13 ± 3	11 ± 0	48 ± 6	120 ± 8	284 ± 20	429 ± 12
	Metalaxyl-M/	33 µg	12 ± 2	13 ± 3	45 ± 13	113 ± 17	261 ± 17	408 ± 27
	Cyclobutrifluram	100 µg	10 ± 4	13 ± 3	44 ± 7	120 ± 19	276 ± 43	361 ± 13
	FS (A23793B)	333 µg	12 ± 2	13 ± 1	50 ± 7	144 ± 17	281 ± 27	417 ± 47
		1000 µg	9 ± 2	14 ± 2	32 ± 5	133 ± 8	235 ± 13	334 ± 11
		2500 µg	11 ± 2	10 ± 1	31 ± 5	118 ± 17	193 ± 18	295 ± 30
		5000 µg	8 ± 1 [P]	10 ± 2 [P]	27 ± 6 [P]	118 ± 9 [P]	179 ± 17 [P]	273 ± 11 [P]
	2-AA	2.5 µg	287 ± 14	373 ± 26	2162 ± 35	3482 ± 135		
	2-AA	10.0 µg					963 ± 17	1414 ± 8

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Key to Plate Postfix Codes

P	Precipitate

TABLE 2 Summary of Results Experiment II

Study Name: 2190200
Experiment: 2190200 HV2 Pre
Assay Conditions:

Study Code: ICCR 2190200
Date Plated: 12.01.2022
Date Counted: 19.01.2022

Metabolic Activation	Test Group	Concentration (per plate)	Revertant Colony Counts (Mean ±SD)					
			TA 1535	TA 1537	TA 98	TA 100	WP2 pKM101	WP2 uvrA pKM101
Without Activation	DMSO		11 ± 4	12 ± 4	27 ± 5	118 ± 14	296 ± 4	359 ± 6
	Untreated		12 ± 4	13 ± 4	30 ± 4	126 ± 5	343 ± 45	402 ± 4
	Difenoconazole/	33 µg	13 ± 1	10 ± 4	28 ± 3	112 ± 14	317 ± 16	374 ± 4
	Fludioxonil/	100 µg	11 ± 1	10 ± 1	24 ± 7	114 ± 13	308 ± 16	352 ± 22
	Metalaxyl-M/	333 µg	10 ± 4	12 ± 2	27 ± 5	128 ± 7	263 ± 19	310 ± 6
	Cyclobutrifluram	1000 µg	13 ± 1	12 ± 4	17 ± 1	108 ± 18	243 ± 3	326 ± 27
	FS (A23793B)	2500 µg	8 ± 3	10 ± 3	23 ± 4	104 ± 5	199 ± 28	278 ± 12
		5000 µg	10 ± 4 [P]	7 ± 2 [P M]	13 ± 2 [P M]	73 ± 13 [P M]	122 ± 14 [P]	249 ± 16 [P]
	NaN3	10 µg	1136 ± 59			1907 ± 30		
	4-NOPD	10 µg			636 ± 24			
	4-NOPD	50 µg		105 ± 32				
	MMS	2.0 µL					3410 ± 143	3301 ± 68
With Activation	DMSO		12 ± 2	13 ± 3	34 ± 5	99 ± 24	315 ± 8	462 ± 17
	Untreated		14 ± 2	14 ± 3	50 ± 1	104 ± 6	344 ± 2	474 ± 15
	Difenoconazole/	33 µg	15 ± 1	16 ± 1	40 ± 9	97 ± 16	319 ± 7	476 ± 9
	Fludioxonil/	100 µg	14 ± 2	15 ± 4	43 ± 3	106 ± 11	316 ± 11	468 ± 14
	Metalaxyl-M/	333 µg	14 ± 3	16 ± 2	27 ± 5	119 ± 8	309 ± 7	379 ± 28
	Cyclobutrifluram	1000 µg	9 ± 2	18 ± 2	34 ± 3	127 ± 2	292 ± 27	387 ± 20
	FS (A23793B)	2500 µg	13 ± 1	18 ± 3	25 ± 8	107 ± 6	238 ± 11	320 ± 23
		5000 µg	11 ± 3 [P]	10 ± 1 [P M]	17 ± 6 [P M]	71 ± 9 [P M]	205 ± 13 [P]	292 ± 10 [P]
	2-AA	2.5 µg	306 ± 30	332 ± 8	2907 ± 96	2903 ± 280		
	2-AA	10.0 µg					973 ± 29	1668 ± 61

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

TABLE 3 Pre-Experiment/Experiment I: 2190200 VV Plate Incorporation Without Metabolic Activation

Study Name: 2190200
Experiment: 2190200 VV Plate
Assay Conditions:

Study Code: ICCR 2190200
Date Plated: 14.12.2021
Date Counted: 17.12.2021

Without metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Difenoconazole/**	3 µg	*11.0*	*1.0*	*1.0*	11, 10, 12
	Fludioxonil/	10 µg	*15.3*	*1.5*	*1.4*	17, 15, 14
	Metalaxyl-M/	33 µg	*13.7*	*2.5*	*1.2*	11, 16, 14
	Cyclobutrifluram	100 µg	*11.0*	*3.6*	*1.0*	12, 7, 14
	FS (A23793B)	333 µg	*12.0*	*4.4*	*1.1*	10, 9, 17
		1000 µg	*9.7*	*2.5*	*0.9*	12, 10, 7
		2500 µg	*10.0*	*2.6*	*0.9*	12, 11, 7
		5000 µg	*8.7*	*2.5*	*0.8*	11 P, 6 P, 9 P
	DMSO		*11.3*	*3.2*		10, 9, 15
	Untreated		*13.0*	*1.7*		12, 12, 15
TA 1537	**Difenoconazole/**	3 µg	*12.0*	*0.0*	*1.2*	12, 12, 12
	Fludioxonil/	10 µg	*11.3*	*3.2*	*1.1*	10, 9, 15
	Metalaxyl-M/	33 µg	*9.0*	*2.0*	*0.9*	7, 9, 11
	Cyclobutrifluram	100 µg	*9.3*	*2.5*	*0.9*	12, 9, 7
	FS (A23793B)	333 µg	*11.7*	*2.1*	*1.2*	14, 10, 11
		1000 µg	*10.0*	*2.6*	*1.0*	11, 12, 7
		2500 µg	*6.3*	*1.2*	*0.6*	5, 7, 7
		5000 µg	*10.0*	*1.0*	*1.0*	9 P, 11 P, 10 P
	DMSO		*10.0*	*3.6*		9, 7, 14
	Untreated		*9.7*	*1.2*		9, 9, 11
TA 98	**Difenoconazole/**	3 µg	*27.0*	*8.7*	*0.8*	31, 33, 17
	Fludioxonil/	10 µg	*33.3*	*8.5*	*1.0*	33, 25, 42
	Metalaxyl-M/	33 µg	*36.3*	*4.2*	*1.1*	35, 33, 41
	Cyclobutrifluram	100 µg	*25.7*	*5.0*	*0.8*	21, 31, 25
	FS (A23793B)	333 µg	*22.7*	*7.4*	*0.7*	31, 20, 17
		1000 µg	*30.0*	*10.1*	*0.9*	28, 21, 41
		2500 µg	*25.7*	*8.1*	*0.8*	17, 27, 33
		5000 µg	*23.3*	*7.1*	*0.7*	31 P, 17 P, 22 P
	DMSO		*32.7*	*2.9*		31, 36, 31
	Untreated		*30.7*	*5.5*		25, 31, 36

Key to Plate Postfix Codes

P Precipitate

Study Name: 2190200
Experiment: 2190200 VV Plate
Assay Conditions:

Study Code: ICCR 2190200
Date Plated: 14.12.2021
Date Counted: 17.12.2021

Without metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 100	**Difenoconazole/**	3 µg	*124.0*	*6.1*	*1.0*	128, 117, 127
	Fludioxonil/	10 µg	*123.0*	*16.6*	*1.0*	116, 142, 111
	Metalaxyl-M/	33 µg	*124.7*	*9.6*	*1.0*	116, 123, 135
	Cyclobutrifluram	100 µg	*136.3*	*8.6*	*1.1*	144, 138, 127
	FS (A23793B)	333 µg	*124.0*	*18.0*	*1.0*	119, 109, 144
		1000 µg	*117.7*	*15.9*	*0.9*	136, 110, 107
		2500 µg	*130.3*	*4.9*	*1.0*	128, 127, 136
		5000 µg	*120.0*	*9.5*	*1.0*	109 P, 125 P, 126 P
	DMSO		*124.3*	*15.3*		111, 141, 121
	Untreated		*127.7*	*10.8*		140, 123, 120
WP2 pKM101	**Difenoconazole/**	3 µg	*283.0*	*28.1*	*1.0*	310, 285, 254
	Fludioxonil/	10 µg	*260.7*	*3.2*	*0.9*	263, 257, 262
	Metalaxyl-M/	33 µg	*272.7*	*14.7*	*0.9*	284, 278, 256
	Cyclobutrifluram	100 µg	*239.7*	*8.3*	*0.8*	233, 237, 249
	FS (A23793B)	333 µg	*273.3*	*10.1*	*0.9*	268, 285, 267
		1000 µg	*228.7*	*6.7*	*0.8*	233, 221, 232
		2500 µg	*209.0*	*21.9*	*0.7*	228, 214, 185
		5000 µg	*163.0*	*20.7*	*0.6*	140 P, 169 P, 180 P
	DMSO		*294.3*	*1.5*		294, 293, 296
	Untreated		*322.0*	*27.0*		309, 304, 353
WP2 uvrA pKM101	**Difenoconazole/**	3 µg	*388.3*	*14.0*	*1.2*	403, 387, 375
	Fludioxonil/	10 µg	*304.7*	*17.9*	*0.9*	316, 284, 314
	Metalaxyl-M/	33 µg	*296.0*	*16.1*	*0.9*	291, 314, 283
	Cyclobutrifluram	100 µg	*327.0*	*39.4*	*1.0*	299, 310, 372
	FS (A23793B)	333 µg	*326.3*	*23.1*	*1.0*	353, 312, 314
		1000 µg	*280.0*	*32.1*	*0.9*	273, 315, 252
		2500 µg	*258.3*	*28.4*	*0.8*	279, 270, 226
		5000 µg	*222.7*	*21.2*	*0.7*	226 P, 242 P, 200 P
	DMSO		*324.7*	*11.0*		316, 337, 321
	Untreated		*359.3*	*3.2*		363, 357, 358
TA 1535	**NaN3**	10 µg	*1175.0*	*46.9*	*103.7*	1206, 1198, 1121
TA 1537	**4-NOPD**	50 µg	*109.3*	*9.7*	*10.9*	120, 101, 107
TA 98	**4-NOPD**	10 µg	*641.0*	*15.1*	*19.6*	653, 646, 624
TA 100	**NaN3**	10 µg	*1673.3*	*105.1*	*13.5*	1747, 1720, 1553
WP2 pKM101	**MMS**	2.0 µL	*2970.0*	*146.2*	*10.1*	2803, 3075, 3032
WP2 uvrA pKM101	**MMS**	2.0 µL	*2898.0*	*168.0*	*8.9*	2898, 3066, 2730

Key to Positive Controls

NaN3	sodium azide
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Key to Plate Postfix Codes

P	Precipitate

TABLE 4 Pre-Experiment/Experiment I: 2190200 VV Plate Incorporation With Metabolic Activation

Study Name: 2190200
Experiment: 2190200 VV Plate
Assay Conditions:

Study Code: ICCR 2190200
Date Plated: 14.12.2021
Date Counted: 17.12.2021

With metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Difenoconazole/**	3 µg	*10.0*	*2.6*	*1.0*	11, 7, 12
	Fludioxonil/	10 µg	*13.0*	*2.6*	*1.3*	14, 15, 10
	Metalaxyl-M/	33 µg	*12.3*	*2.3*	*1.2*	11, 15, 11
	Cyclobutrifluram	100 µg	*10.0*	*4.0*	*1.0*	14, 10, 6
	FS (A23793B)	333 µg	*12.0*	*1.7*	*1.2*	11, 14, 11
		1000 µg	*9.3*	*2.1*	*0.9*	11, 7, 10
		2500 µg	*10.7*	*1.5*	*1.0*	12, 11, 9
		5000 µg	*8.3*	*1.2*	*0.8*	9 P, 9 P, 7 P
	DMSO		*10.3*	*1.5*		10, 12, 9
	Untreated		*14.0*	*2.6*		11, 15, 16
TA 1537	**Difenoconazole/**	3 µg	*12.3*	*2.3*	*0.9*	15, 11, 11
	Fludioxonil/	10 µg	*11.0*	*0.0*	*0.8*	11, 11, 11
	Metalaxyl-M/	33 µg	*13.0*	*2.6*	*1.0*	10, 14, 15
	Cyclobutrifluram	100 µg	*13.3*	*2.9*	*1.0*	10, 15, 15
	FS (A23793B)	333 µg	*12.7*	*1.2*	*0.9*	12, 14, 12
		1000 µg	*13.7*	*1.5*	*1.0*	12, 15, 14
		2500 µg	*10.3*	*0.6*	*0.8*	11, 10, 10
		5000 µg	*10.3*	*1.5*	*0.8*	9 P, 10 P, 12 P
	DMSO		*13.7*	*2.5*		14, 16, 11
	Untreated		*13.3*	*3.1*		16, 10, 14
TA 98	**Difenoconazole/**	3 µg	*41.7*	*12.1*	*0.9*	28, 51, 46
	Fludioxonil/	10 µg	*48.0*	*6.0*	*1.1*	54, 48, 42
	Metalaxyl-M/	33 µg	*45.0*	*13.0*	*1.0*	30, 53, 52
	Cyclobutrifluram	100 µg	*44.0*	*6.6*	*1.0*	38, 43, 51
	FS (A23793B)	333 µg	*49.7*	*7.1*	*1.1*	56, 51, 42
		1000 µg	*31.7*	*4.7*	*0.7*	28, 37, 30
		2500 µg	*30.7*	*5.0*	*0.7*	26, 30, 36
		5000 µg	*27.0*	*6.0*	*0.6*	27 P, 21 P, 33 P
	DMSO		*45.0*	*13.5*		56, 30, 49
	Untreated		*45.7*	*2.3*		43, 47, 47

Key to Plate Postfix Codes

P Precipitate

Study Name: 2190200
Experiment: 2190200 VV Plate
Assay Conditions:

Study Code: ICCR 2190200
Date Plated: 14.12.2021
Date Counted: 17.12.2021

With metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 100	**Difenoconazole/**	3 µg	*104.3*	*0.6*	*0.8*	104, 105, 104
	Fludioxonil/	10 µg	*119.7*	*7.8*	*0.9*	111, 126, 122
	Metalaxyl-M/	33 µg	*113.3*	*17.0*	*0.8*	120, 126, 94
	Cyclobutrifluram	100 µg	*120.3*	*18.9*	*0.9*	104, 116, 141
	FS (A23793B)	333 µg	*143.7*	*16.6*	*1.1*	159, 126, 146
		1000 µg	*132.7*	*8.0*	*1.0*	132, 125, 141
		2500 µg	*118.3*	*17.0*	*0.9*	117, 102, 136
		5000 µg	*118.0*	*8.5*	*0.9*	126 P, 109 P, 119 P
	DMSO		*134.3*	*12.4*		120, 141, 142
	Untreated		*128.3*	*13.7*		116, 143, 126
WP2 pKM101	**Difenoconazole/**	3 µg	*304.0*	*5.6*	*1.1*	309, 298, 305
	Fludioxonil/	10 µg	*284.3*	*20.3*	*1.0*	298, 294, 261
	Metalaxyl-M/	33 µg	*261.3*	*16.8*	*0.9*	242, 270, 272
	Cyclobutrifluram	100 µg	*276.0*	*43.1*	*1.0*	235, 272, 321
	FS (A23793B)	333 µg	*280.7*	*26.5*	*1.0*	311, 262, 269
		1000 µg	*234.7*	*13.1*	*0.8*	247, 236, 221
		2500 µg	*192.7*	*17.9*	*0.7*	172, 203, 203
		5000 µg	*179.3*	*16.5*	*0.6*	163 P, 179 P, 196 P
	DMSO		*286.3*	*8.5*		280, 283, 296
	Untreated		*340.0*	*8.9*		330, 347, 343
WP2 uvrA pKM101	**Difenoconazole/**	3 µg	*469.0*	*55.7*	*1.2*	522, 474, 411
	Fludioxonil/	10 µg	*429.3*	*12.0*	*1.1*	441, 430, 417
	Metalaxyl-M/	33 µg	*407.7*	*26.6*	*1.0*	377, 422, 424
	Cyclobutrifluram	100 µg	*361.0*	*13.0*	*0.9*	346, 369, 368
	FS (A23793B)	333 µg	*417.0*	*46.6*	*1.0*	469, 403, 379
		1000 µg	*334.0*	*10.5*	*0.8*	333, 324, 345
		2500 µg	*295.3*	*29.7*	*0.7*	288, 328, 270
		5000 µg	*273.0*	*11.1*	*0.7*	261 P, 275 P, 283 P
	DMSO		*398.3*	*31.3*		433, 390, 372
	Untreated		*397.3*	*29.1*		430, 374, 388
TA 1535	**2-AA**	2.5 µg	*287.3*	*13.8*	*27.8*	303, 282, 277
TA 1537	**2-AA**	2.5 µg	*373.3*	*25.7*	*27.3*	359, 358, 403
TA 98	**2-AA**	2.5 µg	*2162.3*	*35.2*	*48.1*	2187, 2178, 2122
TA 100	**2-AA**	2.5 µg	*3482.3*	*135.0*	*25.9*	3398, 3638, 3411
WP2 pKM101	**2-AA**	10.0 µg	*962.7*	*17.0*	*3.4*	945, 979, 964
WP2 uvrA pKM101	**2-AA**	10.0 µg	*1414.3*	*7.6*	*3.6*	1409, 1423, 1411

Key to Positive Controls		Key to Plate Postfix Codes	
2-AA	2-aminoanthracene	P	Precipitate

TABLE 5 Experiment II: 2190200 HV2 Pre Incubation Without Metabolic Activation

Study Name: 2190200
Experiment: 2190200 HV2 Pre
Assay Conditions:

Study Code: ICCR 2190200
Date Plated: 12.01.2022
Date Counted: 19.01.2022

Without metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Difenoconazole/**	33 µg	*13.3*	*1.2*	*1.3*	12, 14, 14
	Fludioxonil/	100 µg	*10.7*	*0.6*	*1.0*	10, 11, 11
	Metalaxyl-M/	333 µg	*10.0*	*3.6*	*0.9*	9, 7, 14
	Cyclobutrifluram	1000 µg	*12.7*	*1.2*	*1.2*	14, 12, 12
	FS (A23793B)	2500 µg	*8.3*	*3.2*	*0.8*	6, 12, 7
		5000 µg	*10.3*	*3.5*	*1.0*	10 P, 14 P, 7 P
	DMSO		*10.7*	*3.5*		14, 7, 11
	Untreated		*12.0*	*4.4*		10, 9, 17
TA 1537	**Difenoconazole/**	33 µg	*10.0*	*3.6*	*0.9*	7, 9, 14
	Fludioxonil/	100 µg	*10.0*	*1.0*	*0.9*	10, 9, 11
	Metalaxyl-M/	333 µg	*12.3*	*2.3*	*1.1*	11, 15, 11
	Cyclobutrifluram	1000 µg	*11.7*	*3.8*	*1.0*	10, 16, 9
	FS (A23793B)	2500 µg	*9.7*	*3.2*	*0.8*	6, 11, 12
		5000 µg	*7.3*	*2.1*	*0.6*	9 P M, 8 P M, 5 P M
	DMSO		*11.7*	*4.0*		14, 14, 7
	Untreated		*13.0*	*3.6*		16, 9, 14
TA 98	**Difenoconazole/**	33 µg	*28.0*	*3.0*	*1.0*	25, 28, 31
	Fludioxonil/	100 µg	*24.3*	*6.7*	*0.9*	21, 20, 32
	Metalaxyl-M/	333 µg	*26.7*	*4.5*	*1.0*	31, 22, 27
	Cyclobutrifluram	1000 µg	*16.7*	*0.6*	*0.6*	16, 17, 17
	FS (A23793B)	2500 µg	*22.7*	*4.0*	*0.8*	27, 22, 19
		5000 µg	*12.7*	*2.1*	*0.5*	11 P M, 15 P M, 12 P M
	DMSO		*27.3*	*5.1*		23, 33, 26
	Untreated		*30.3*	*4.0*		35, 28, 28
TA 100	**Difenoconazole/**	33 µg	*111.7*	*14.4*	*0.9*	95, 120, 120
	Fludioxonil/	100 µg	*114.3*	*13.0*	*1.0*	115, 101, 127
	Metalaxyl-M/	333 µg	*128.3*	*6.8*	*1.1*	123, 136, 126
	Cyclobutrifluram	1000 µg	*107.7*	*17.7*	*0.9*	96, 99, 128
	FS (A23793B)	2500 µg	*104.0*	*5.3*	*0.9*	100, 110, 102
		5000 µg	*73.0*	*13.0*	*0.6*	65 P M, 88 P M, 66 P M
	DMSO		*118.0*	*13.9*		102, 125, 127
	Untreated		*126.0*	*5.0*		121, 126, 131

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

Study Name: 2190200
Experiment: 2190200 HV2 Pre
Assay Conditions:

Study Code: ICCR 2190200
Date Plated: 12.01.2022
Date Counted: 19.01.2022

Without metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
WP2	**Difenoconazole/**	33 µg	*317.0*	*16.5*	*1.1*	327, 326, 298
pKM101	**Fludioxonil/**	100 µg	*308.0*	*15.6*	*1.0*	326, 300, 298
	Metalaxyl-M/	333 µg	*263.0*	*19.3*	*0.9*	259, 246, 284
	Cyclobutrifluram	1000 µg	*243.0*	*2.6*	*0.8*	241, 246, 242
	FS (A23793B)	2500 µg	*198.7*	*28.2*	*0.7*	186, 231, 179
		5000 µg	*121.7*	*13.6*	*0.4*	111 P, 137 P, 117 P
	DMSO		*296.3*	*3.5*		296, 293, 300
	Untreated		*343.0*	*45.1*		395, 314, 320
WP2	**Difenoconazole/**	33 µg	*374.0*	*4.0*	*1.0*	370, 374, 378
uvrA	**Fludioxonil/**	100 µg	*351.7*	*22.3*	*1.0*	377, 335, 343
pKM101	**Metalaxyl-M/**	333 µg	*310.0*	*5.6*	*0.9*	304, 315, 311
	Cyclobutrifluram	1000 µg	*325.7*	*27.4*	*0.9*	294, 341, 342
	FS (A23793B)	2500 µg	*278.0*	*12.5*	*0.8*	288, 264, 282
		5000 µg	*249.0*	*16.0*	*0.7*	265 P, 249 P, 233 P
	DMSO		*359.3*	*5.7*		364, 361, 353
	Untreated		*402.3*	*3.8*		398, 404, 405
TA 1535	**NaN3**	10 µg	*1135.7*	*58.8*	*106.5*	1202, 1115, 1090
TA 1537	**4-NOPD**	50 µg	*105.3*	*32.0*	*9.0*	137, 106, 73
TA 98	**4-NOPD**	10 µg	*636.3*	*23.5*	*23.3*	612, 659, 638
TA 100	**NaN3**	10 µg	*1907.0*	*30.1*	*16.2*	1881, 1940, 1900
WP2 pKM101	**MMS**	2.0 µL	*3410.3*	*142.5*	*11.5*	3537, 3438, 3256
WP2 uvrA pKM101	**MMS**	2.0 µL	*3301.0*	*67.8*	*9.2*	3316, 3227, 3360

Key to Positive Controls

NaN3	sodium azide
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Key to Plate Postfix Codes

P	Precipitate

TABLE 6 Experiment II: 2190200 HV2 Pre Incubation With Metabolic Activation

Study Name: 2190200
Experiment: 2190200 HV2 Pre
Assay Conditions:

Study Code: ICCR 2190200
Date Plated: 12.01.2022
Date Counted: 19.01.2022

With metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Difenoconazole/**	33 µg	*15.3*	*0.6*	*1.2*	15, 15, 16
	Fludioxonil/	100 µg	*14.3*	*2.1*	*1.2*	15, 16, 12
	Metalaxyl-M/	333 µg	*14.3*	*3.1*	*1.2*	15, 17, 11
	Cyclobutrifluram	1000 µg	*8.7*	*1.5*	*0.7*	7, 10, 9
	FS (A23793B)	2500 µg	*13.3*	*1.2*	*1.1*	14, 12, 14
		5000 µg	*10.7*	*2.9*	*0.9*	14 P, 9 P, 9 P
	DMSO		*12.3*	*2.3*		11, 15, 11
	Untreated		*13.7*	*2.3*		15, 11, 15
TA 1537	**Difenoconazole/**	33 µg	*16.0*	*1.0*	*1.2*	17, 15, 16
	Fludioxonil/	100 µg	*15.3*	*4.0*	*1.2*	11, 19, 16
	Metalaxyl-M/	333 µg	*16.3*	*2.3*	*1.3*	15, 19, 15
	Cyclobutrifluram	1000 µg	*18.0*	*1.7*	*1.4*	16, 19, 19
	FS (A23793B)	2500 µg	*18.3*	*3.1*	*1.4*	19, 21, 15
		5000 µg	*10.0*	*1.0*	*0.8*	10 P M, 11 P M, 9 P M
	DMSO		*13.0*	*2.6*		14, 15, 10
	Untreated		*14.3*	*2.5*		14, 17, 12
TA 98	**Difenoconazole/**	33 µg	*40.3*	*9.0*	*1.2*	31, 49, 41
	Fludioxonil/	100 µg	*42.7*	*3.1*	*1.3*	42, 46, 40
	Metalaxyl-M/	333 µg	*27.3*	*4.5*	*0.8*	32, 23, 27
	Cyclobutrifluram	1000 µg	*34.3*	*3.1*	*1.0*	37, 31, 35
	FS (A23793B)	2500 µg	*25.3*	*8.1*	*0.7*	16, 30, 30
		5000 µg	*17.3*	*5.9*	*0.5*	15 P M, 13 P M, 24 P M
	DMSO		*34.0*	*5.3*		32, 40, 30
	Untreated		*49.7*	*1.2*		49, 51, 49

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

Study Name: 2190200
Experiment: 2190200 HV2 Pre
Assay Conditions:

Study Code: ICCR 2190200
Date Plated: 12.01.2022
Date Counted: 19.01.2022

With metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 100	**Difenoconazole/**	33 µg	*96.7*	*15.9*	*1.0*	110, 101, 79
	Fludioxonil/	100 µg	*106.0*	*11.4*	*1.1*	119, 98, 101
	Metalaxyl-M/	333 µg	*119.0*	*7.5*	*1.2*	111, 126, 120
	Cyclobutrifluram	1000 µg	*126.7*	*1.5*	*1.3*	125, 128, 127
	FS (A23793B)	2500 µg	*107.0*	*6.2*	*1.1*	100, 112, 109
		5000 µg	*70.7*	*9.1*	*0.7*	64 P M, 81 P M, 67 P M
	DMSO		*98.7*	*24.0*		98, 75, 123
	Untreated		*104.3*	*6.0*		110, 105, 98
WP2 pKM101	**Difenoconazole/**	33 µg	*319.0*	*7.0*	*1.0*	314, 316, 327
	Fludioxonil/	100 µg	*316.3*	*11.0*	*1.0*	320, 325, 304
	Metalaxyl-M/	333 µg	*308.7*	*7.4*	*1.0*	317, 303, 306
	Cyclobutrifluram	1000 µg	*292.3*	*27.1*	*0.9*	301, 314, 262
	FS (A23793B)	2500 µg	*238.0*	*10.6*	*0.8*	246, 242, 226
		5000 µg	*204.7*	*12.9*	*0.6*	194 P, 201 P, 219 P
	DMSO		*315.0*	*7.5*		322, 307, 316
	Untreated		*343.7*	*2.3*		341, 345, 345
WP2 uvrA pKM101	**Difenoconazole/**	33 µg	*476.0*	*8.7*	*1.0*	466, 482, 480
	Fludioxonil/	100 µg	*468.3*	*13.6*	*1.0*	473, 453, 479
	Metalaxyl-M/	333 µg	*379.0*	*28.1*	*0.8*	352, 377, 408
	Cyclobutrifluram	1000 µg	*386.7*	*19.7*	*0.8*	396, 364, 400
	FS (A23793B)	2500 µg	*320.3*	*22.7*	*0.7*	303, 312, 346
		5000 µg	*292.0*	*9.6*	*0.6*	303 P, 288 P, 285 P
	DMSO		*461.7*	*16.7*		475, 467, 443
	Untreated		*473.7*	*14.6*		480, 457, 484
TA 1535	**2-AA**	2.5 µg	*306.3*	*29.6*	*24.8*	337, 278, 304
TA 1537	**2-AA**	2.5 µg	*332.0*	*7.8*	*25.5*	328, 341, 327
TA 98	**2-AA**	2.5 µg	*2907.0*	*96.2*	*85.5*	2980, 2943, 2798
TA 100	**2-AA**	2.5 µg	*2903.3*	*280.2*	*29.4*	3075, 3055, 2580
WP2 pKM101	**2-AA**	10.0 µg	*973.0*	*29.5*	*3.1*	979, 999, 941
WP2 uvrA pKM101	**2-AA**	10.0 µg	*1668.3*	*61.5*	*3.6*	1677, 1725, 1603

Key to Positive Controls

2-AA 2-aminoanthracene

Key to Plate Postfix Codes

P Precipitate
M Manual count

APPENDICES SECTION

APPENDIX 1 Historical Control Data

These data represent the laboratory´s historical control data from July 2018 until July 2020 representing approx. 600 experiments (WP2 pKM101, WP2 uvrA pKM101 the historical data are based on approx. 80 experiments).

The positive controls that used to compile the historical positive control data correspond to the positive control substances described in Methods; section 3.2.2 (Positive control substances).

Strain		without S9 mix				with S9 mix			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA 1535	Solvent control	12	2.6	7	22	13	2.5	7	24
	Untreated control	12	2.9	6	26	13	2.8	7	23
	Positive control	1116	141.3	340	1612	346	72.1	170	736
TA1537	Solvent control	11	2.4	6	20	14	2.8	7	28
	Untreated control	11	2.8	5	22	14	3.2	7	30
	Positive control	83	22.1	48	400	286	98.7	82	630
TA 98	Solvent control	28	4.9	13	46	38	6.4	12	62
	Untreated control	29	5.0	14	48	41	6.8	14	64
	Positive control	421	91.2	216	1218	3275	774.9	322	5699
TA 100	Solvent control	127	30.7	63	214	131	30.0	72	214
	Untreated control	135	35.7	64	233	140	34.4	68	217
	Positive control	1759	273.4	511	2588	3566	837.6	553	5444
WP2 pKM 101	Solvent control	248	31.7	171	299	266	33.0	205	315
	Untreated control	269	26.6	212	346	299	28.2	233	345
	Positive control	3343	428.4	2332	4653	1092	257.8	933	2781
WP2uvrA pKM 101	Solvent control	322	31.6	248	388	375	38.5	287	466
	Untreated control	346	28.2	279	403	393	32.6	313	480
	Positive control	3176	468.5	2021	4717	1897	183.2	1270	2464

Mean = mean value of revertants/plate
SD = standard deviation
Min = minimal value
Max = maximal value

APPENDIX 2 Copy of GLP Certificate

HESSEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

ICCR-Roßdorf GmbH
Institute for Competent Contract Research
In den Leppsteinswiesen 19
64380 Roßdorf

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß according ChemVwV-GLP Nr. 5.3/OECD guidance)

2 Prüfungen zur Bestimmung der toxikologischen Eigenschaften	2 Toxicity studies
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)	3 Mutagenicity studies
8 Analytische Prüfungen an biologischen Materialien	8 Analytical and clinical chemistry testing

22.11.2018, 21.02.2019, 12. bis 14.03.2019
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltuog der GLP- Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag

Brandt

Dr. Astrid Brandt, Referentin, Wiesbaden, den **23. Oktober 2019**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, KLIMASCHUTZ, LANDWIRTSCHAFT UND VERBRAUCHERSCHUTZ

Hessisches Ministerium für Umwelt, Klimaschutz, Landwirtschaft und Verbraucherschutz,
Mainzer Straße 80, D 65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority)

English name and address of the GLP Monitoring Authority: Hessian Ministry for Environment, Climate Protection, Agriculture and Consumer Protection; Department II 10; P.O. Box 31 09; 65189 Wiesbaden
Translation of seal inscription: Hessian Ministry for Environment, Climate Protection, Agriculture and Consumer Protection

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APPENDIX 3 Certificate of S9

ICCR ROßDORF

CERTIFICATE

ICCR-Roßdorf S9 Preparation Lot No. 080721D

Date of preparation: July 08, 2021
Release date: July 26, 2021

Protein assay: 29.6 mg protein / ml S9

Sterility: 7.6 colonies / ml S9 on glucose-minimal-agar

Salmonella typhimurium assay (AMES-test)

Treatment	µl S9 / plate	number of revertants in TA 98
negative	0	33
control	100	54
10 µg/plate	0	40
2-Aminoanthracene	100	2031
10 µg/plate	0	23
Benzo(a)pyrene	100	88

The S9 was obtained from the livers of male Wistar rats which received triple treatments of 80 mg / kg body weight Phenobarbital and ß-Naphthoflavone orally on consecutive days. The livers were prepared 24 hours after the last treatment.

S. Ebert Sabine Ebert — 27. JULI 2021

Quality Assurance Auditor
ICCR-Roßdorf GmbH — Date

27. JULI 2021

Dr. Steffen Naumann
Study Director
ICCR-Roßdorf GmbH — Date

ICCR-Roßdorf GmbH
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Registergericht Darmstadt, HRB 6837, USt.-ID DE812333696
Geschäftsführer: Dr. Markus Schulz

SOP Origin TS-SOP S9_23

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APPENDIX 4 Certificate of Analysis

syngenta

Syngenta Crop Protection, LLC
Analytical and Product Chemistry
Greensboro, NC 27409

Certificate of Analysis

A23793B

Batch ID 1200767 (GP210610)

Test Substance Name:	CGA169374/CGA173506/CGA329351/SYN549522 FS (062.51/049.93/050.05/250.08)
Common Name:	Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (062.51/049.93/050.05/250.08)
Material ID:	A23793B
Batch ID:	1200767
Other ID:	GP210610
Source:	Syngenta Crop Protection LLC., 410 Swing Road, Greensboro, NC 27409, US

Chemical Analysis

AI	% w/w	g/L
Difenoconazole	5.45	64.0
Fludioxonil	4.37	51.3
Metalaxyl-M	4.31	50.6
Cyclobutrifluram	21.0	247

Identity of the Active Ingredients: Confirmed

Methodology Used for Characterization: LC, mass spectrometry, oscillating density meter.

The Active Ingredient(s) content is within the FAO limits.

Isomer Assay

AI	Isomer	% w/w
CGA329351	D-alanine, N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-, Methyl Ester	4.15
CGA351920	L-alanine, N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-, Methyl Ester	0.15

COA Number: USGR210208

Page 1 of 2

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Physical Analysis

Analyte	Value	Units
Density	1.174	g/cm3

Appearance: red liquid

Storage Temperature: <30°C

Re-certification Date:End of Aug 2024

If stored under the conditions given above, this test substance can be considered stable until the recertification date is reached.

The stability of this test substance will be determined concurrently through reanalysis of material held in inventory under GLP conditions at Syngenta Crop Protection, LLC, Greensboro, NC.

This Certificate of Analysis is summarizing data from a study that has been performed in compliance with Good Laboratory Practices per 40 CFR Part 160. Raw data, documentation, protocols, any amendments to study protocols and reports pertaining to this study are maintained in the Syngenta Crop Protection Archives in Greensboro, NC.

Study Number: USGR210208

Authorization: Sherry Perine

Sherry C Perine

Sherry Perine

Analytical and Product Chemistry Department

Aug 24, 2021

Date

COA Number: USGR210208

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