Baseline sensitivity of *Phytophthora infestans* lifecycle components to NC–224 20SC (Amisulbrom 200 g/l)

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Summary
The aim of this study was to determine EC_{50} values of NC-224 20SC (active ingredient amisulbrom (ISO proposed), a new fungicide introduced by Nissan Chemicals Industries, Ltd.) for four stages in the life cycle of *Phytophthora infestans*. The four stages selected were the release of zoospores, motility of zoospores, germination of cystospores and the formation of oospores in planta. The EC_{50} of NC-224 20 SC for zoospore release, motility of zoospores and germination of cystospores was found to be 0.016 ppm, 0.0002 ppm and 0.061 ppm. Oospore formation also responds sensitive to exposure to NC–224 20SC. Both, the total number of oospores and the number of viable oospores formed are reduced. The EC_{50} value for the fraction of viable oospores formed was determined to be 35% of the recommended dose rate.

Keywords
Phytophthora infestans, Amisulbrom, NC-224 20SC, zoospore release, zoospore motility, cystospore germination, oospore formation.

Introduction

Amisulbrom is a new fungicide introduced by Nissan Chemicals Industries, Ltd. Currently, the company is introducing 'NC-224 20SC', a suspension concentrate containing 200g a.i./L amisulbrom, for use on potatoes for the control of late blight caused by *Phytophthora infestans*.

The aim of this study was to determine EC_{50} values of NC-224 20SC for four stages in the life cycle of *Phytophthora infestans*, the causal organism of late blight on potato and tomato. The four selected stages in the *P. infestans* life cycle were: 1) the release of zoospores, 2) motility of zoospores, 3) germination of cystospores and 4) the formation of oospores in planta. The EC_{50} value is defined as the NC-224 20SC concentration or dose rate at which 50% inhibition of the process studied is obtained relative to the control treatment. This study was carried out for Nissan Chemical Industries, Ltd.

Posters

The life cycle of *P. infestans* can be separated into an asexual cycle and a sexual cycle. The asexual cycle is completed many times during the potato growing season. Sporangia are formed and dispersed and germinate directly or indirectly. Direct germination results in formation of a germ tube and potentially infection. Indirect germination results in formation of motile zoospores. When the zoospores loose their flagellae, they become cystospores which germinate and infect through a germ tube.
The sexual cycle is completed only once per growing season. Oospores are formed in host tissue infected by both, the A1 and A2 mating type.

Materials and methods
Two types of experiments were carried out: an in vitro experiment aimed to determine the EC_{50} of NC-224 20SC for the release of zoospores and germination of cystospores in vitro. Furthermore, this experiment aimed to approximate the EC_{50} of NC-224 20SC for zoospore motility in vitro. The second experiment was an in planta experiment to determine the EC_{50} of NC-224 20SC for oospore formation in potato foliage.

In vitro experiment
Sporangia in suspension were exposed to a dilution series of NC-224 20SC introduced at different times during indirect germination. A sporangial suspension (50000 sporangia/ml) of *P. infestans* isolate IPO82001 was obtained by rinsing sporulating potato leaves (c.v. Bintje) in tap water of 4°C. NC-224 20SC was added to aliquots of this suspension at three points in time during zoospore release and germination such that a dilution series for each of the three target processes was created at final concentrations of: 0, 0.001, 0.01, 0.03, 0.1 and 1.0 ppm. The points in time were:
1) From the start (targeting zoospore release)
2) After 2 hours (targeting zoospore motility)
3) After 4 hours (targeting cystospore germination)

Zoospore release (% sporangia releasing zoospores) was determined after 2 hours incubation in the presence of NC-224 20SC at 10°C by microscopically assessing approximately 100 zoosporangia for the release of zoospores (sporangia are empty when zoospores have been released).
Zoospore motility was assessed after 45 minutes incubation at 10°C in the presence of NC-224 20SC and classified as follows:
+: zoospore motility is not inhibited as compared to the control treatment.
+/-: zoospores motility is partially inhibited as compared to the control treatment,
-: zoospore motility is completely inhibited as compared to the control treatment.
In addition it was found possible to count motile and non-motile zoospores. Zoospore motility was therefore also determined by microscopically assessing approximately 100 zoospores for their motility (motile or non-motile).
For cystospore germination, the zoospore suspension was plated on 1.5% water agar plates 15 minutes after adding NC-224 20SC. Germination was assessed after a total of 20 hours incubation in the presence of NC-224 20SC at 10°C by microscopically assessing 100 cystospores for germination.

Statistically, this experiment was set up as completely randomized experiments with 6 concentrations of NC-224 20SC, 3 response variables (zoospore release, zoospore motility and cystospore germination) and 3 replicates.

In planta experiment
Potted potato plants (c.v. Bintje, 8 weeks old) were spray inoculated with a 1:1 mixture of *P. infestans* A1/A2 (isolates Kartel and US8 respectively). Symptoms were allowed to develop for eight

days before treatment with NC-224 20SC, at 5 different dose rates equivalent to 100 g a.i./ha (full rate), 50 g a.i./ha (½ rate), 25 g a.i./ha (¼ rate) , 12.5 g a.i./ha (1/8 rate), 0 g/ha and Tattoo C (2.7 l/ha as commercially available).
Disease severity was assessed 0, 8 and 14 days post inoculation. In addition fourteen days post inoculation, 10 leaflets with multiple lesions were picked from each plant and incubated on water agar at 10°C in a climate chamber for 3 weeks to allow completion of oospore formation. Following this incubation, oospores were extracted from the remaining tissue using the methods described by van Bekkum and Kessel elsewhere in this volume, stained using tetrazolium bromide (Sigma M-2128) and quantified, including a differentiation between live and dead oospores (Jiang and Erwin, 1990).
Statistically, this experiment was set up as a completely randomized experiment including 5 concentrations of NC-224 20SC, 1 response variable (number of viable oospores) and 4 replicates (plants).

Statistical analysis
In vitro experiment:
To allow for a logistic regression analysis, NC-224 20SC concentrations were ^{10}Log transformed and data on zoospore release, zoospore motility and cystospore germination were scaled such that the control treatment (0 ppm NC-224 20SC) represented 100%. A generalized linear model (GLM) for binomially distributed data using the logit function as link function was fitted to these data using Genstat (seventh edition). The EC_{50} value was calculated from the fitted sigmoid curve.

From results it is obvious that zoospore motility is highly sensitive to even the lowest concentration of NC-224 20 SC included in this experiment. From the effect of the dilution series NC-224 20SC on the fraction of motile zoospores it can be derived that the EC_{50} of NC224 20 SC is located in the 0 – 0.001 ppm range. For the statistical analysis this meant the concentration range of NC224 20 SC incorporated in this experiment was not ideally suited to accurately estimate the EC_{50} for zoospore motility.

Oospore formation:
The fraction viable oospores was analyzed through logistic regression analysis using the original (untransformed) NC-224 20SC dose rates and the fraction of viable oospores scaled such that the control treatment (0 ppm NC-224 20SC) represented 100%. A generalized linear model (GLM) for binomially distributed data using the logit function as link function was fitted to these data using Genstat (seventh edition). A comparison of means to test for differences between individual treatment was carried out using a Least Significant Difference (LSD) test at p = 0.05. The EC_{50} value was calculated from the fitted sigmoid curve.

Results
Zoospore release
The sigmoid function, resulting from the logistic regression analysis, describing the effects of a dilution series of NC-224 20SC on the release of zoospores is given in equation 1 and Figure 1.

$$Y = \frac{e^{(-5.567 - 3.122\ X)}}{(1 - e^{(-5.567 - 3.122\ X)})} \qquad \textit{Equation 1}$$

In equation 1, Y represents the proportion of empty sporangia (zoospores released) and X the ^{10}Log of the NC-224 20SC concentration (ppm). This relationship is plotted in Figure 1. The EC_{50} of NC-224 20 SC for zoospore release calculated from this relationship is 0.016 ppm with a standard error of 0.003 ppm.

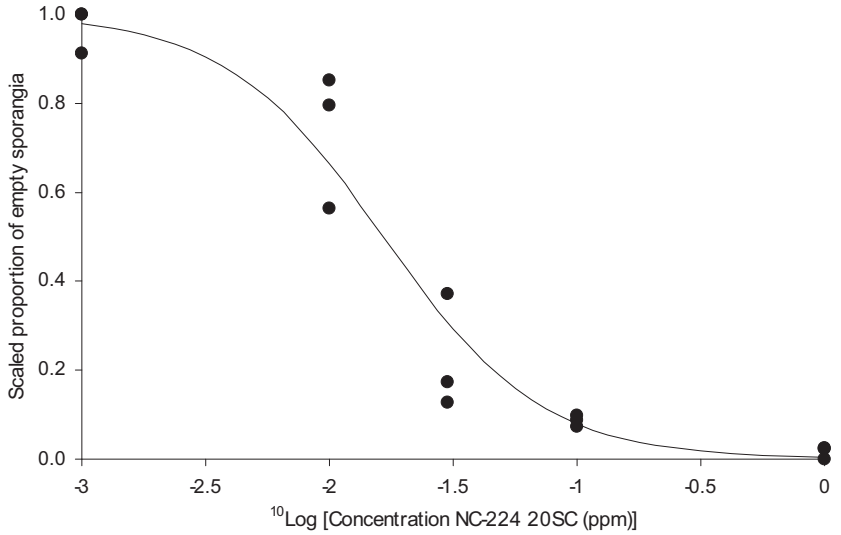


Figure 1. Observations (●) and fitted sigmoid curve (solid line, described by equation 1) describing the relationship between the scaled fraction of empty sporangia (zoospores released) and the ^{10}Log of the concentration of NC-224 20SC (ppm).

Zoospore motility

The sigmoid function, resulting from logistic regression analysis, describing the effects of a dilution series of NC-224 20SC on zoospore motility is given in equation 2 and Figure 2.

$$Y = \frac{e^{(-8.180 - 2.237\ X)}}{(1 - e^{(-8.180 - 2.237\ X)})} \qquad \textit{Equation 2}$$

In equation 2, Y represents the proportion of motile zoospores and X the ^{10}Log of the NC-224 20SC concentration (ppm). This relationship is plotted in Figure 2. Although the EC_{50} of NC-224 20 SC for zoospore release cannot be estimated reliably because it falls outside the range covered by the dilution series used in the experiment, it was calculated to be 0.0002 ppm (0.2 ppb) with a standard error of 0.0001 ppm.

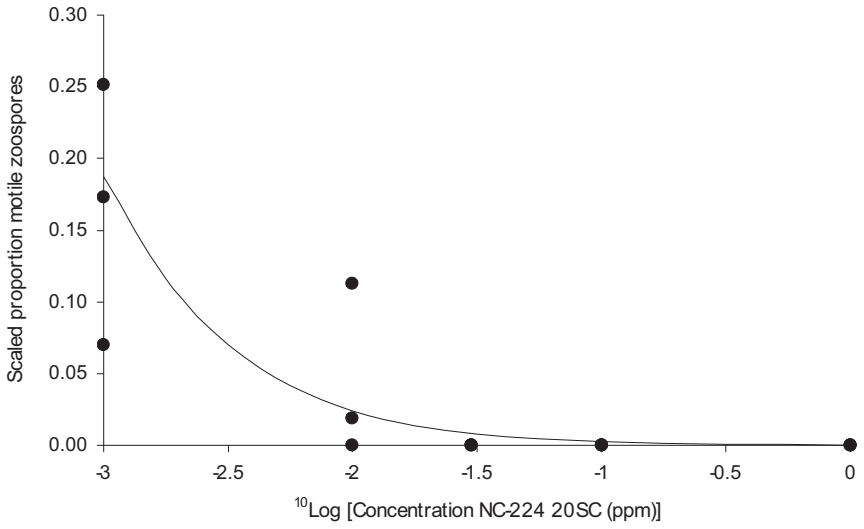


Figure 2. Observations (●) and fitted sigmoid curve (solid line, described by equation 2) describing the relationship between the scaled fraction of motile zoospores and the ^{10}Log of the concentration of NC224 20SC (ppm).

Cystospore germination

The sigmoid function, resulting from logistic regression analysis, describing the effects of a dilution series of NC-224 20SC on cystospore germination is given in equation 3 and Figure 3.

$$Y = \frac{e^{(-2.992 - 2.458\ X)}}{(1 - e^{(-2.992 - 2.458\ X)})} \qquad \textit{Equation 3}$$

In equation 3, Y represents the proportion of germinated cystospores and X the ^{10}Log of the NC-224 20SC concentration (ppm). This relationship is plotted in Figure 3 together with the observations. The EC_{50} of NC-224 20 SC for cystospore germination calculated from this relationship is 0.061 ppm with a standard error of 0.014 ppm.

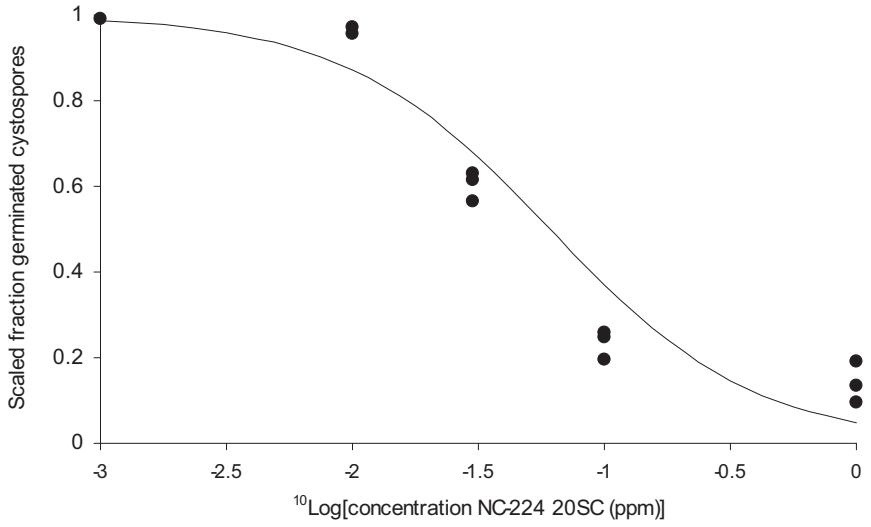


Figure 3. Observations (●) and fitted sigmoid curve (solid line, described by equation 3) describing the relationship between the scaled fraction of germinated cystospores and the ^{10}Log of the concentration of NC-224 20SC (ppm).

Oospore formation in planta

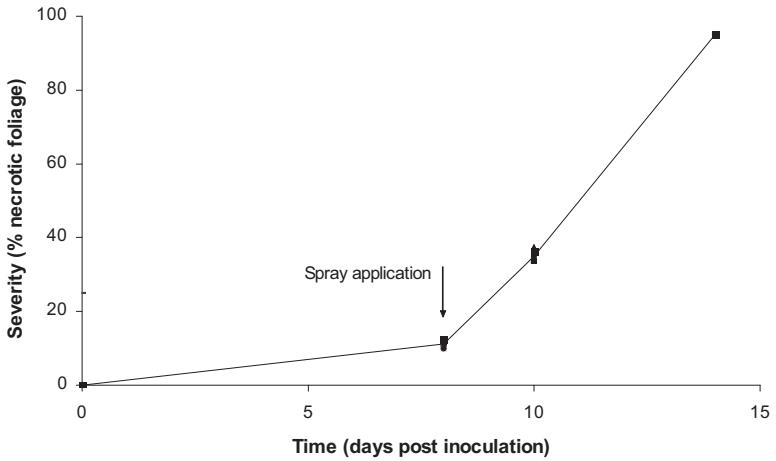
Oospore formation responds sensitive to exposure to NC–224 20SC. Results on the fraction of viable oospores are given in Table 1. Both, the total number of oospores and the number of viable oospores formed are reduced. The EC_{50} value for the fraction of viable oospores was determined to be 35% of the recommended dose rate. NC–224 20SC performed at least equal to Tattoo C, which was included as a reference treatment.

Table 1. Effect of spray treatments using different dose rates of NC-224 20SC, Tattoo C and a water control on the fraction of vital oospores formed in the leaves of infected potato plants. Averages are followed by the standard error of the mean in brackets.

Treatment	Dose rate (% of recommended)	Average fraction viable oospores[1]	Scaled average fraction viable oospores (relative to control treatment)
NC-224 20SC	100%	0.078 a	0.327 (0.111)
Tattoo C [2]	100%	0.093 a	n.d.
NC-224 20SC	50%	0.106 ab	0.448 (0.126)
NC-224 20SC	25%	0.148 ab	0.624 (0.178)
NC-224 20SC	12.5%	0.121 ab	0.540 (0.105)
Control	0	0.237 b	1

1 Statistical analysis was done on original counts in 50µl aliquots of the purified oospore suspension.
2: Tattoo C was applied at 2.7 l/ha as commercially available in the Netherlands.

A



B

Figure 4. A: Development of foliar infection following inoculation at time = 0. Spray applications were carried out eight days after inoculation. B: Potato plants (c.v. Bintje) from the oospore experiment 8 days after inoculation and just prior to treatment with fungicides.

Discussion

In vitro and in vivo experiments were carried out to determine EC_{50} values of NC-224 20SC to four stages in the life cycle of *P. infestans*, the causal organism of potato and tomato late blight. EC_{50} values of NC-224 20 SC for zoospore release, zoospore motility and cystospore germination were determined using logistic regression analysis and found to be 0.016 ppm, 0.0002 ppm and 0.014 ppm respectively.

Oospore formation also responds sensitive to exposure to NC-224 20SC. Both, the total number of oospores and the number of viable oospores formed are reduced by at least 50% and 75% respectively. The EC_{50} value for the fraction of viable oospores was determined to be 35% of the recommended dose rate. When compared to the standard treatments included in the experiment, NC-224 20SC reduces both, the fraction of viable oospores and the total number of oospores formed and performs equally well as Tattoo C. In earlier work, Tattoo C was found to be one of the most effective fungicides

against oospore formation in vivo, currently available on the Dutch market (Kessel *et al.,* 2002).

Overall, NC-224 20SC was found to be very effective against the stages of the *P. infestans* life cycle tested. The sensitivity of sporangia, zoospores and cystospores to NC-224 20SC give reason to believe that this compound could be developed into a fungicide highly effective against spores of *P. infestans.* Protection against foliar and tuber infection are thus likely to be two of the strong points of this new fungicide.

References

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