RESISTANCE RISK EVALUATION OF FLUDIOXONIL, A NEW PHENYLPYRROLE FUNGICIDE

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ABSTRACT

Several authors have dealt with the question whether the resistance risk of new compounds can be assessed in laboratory and greenhouse tests. Risk assessment schemes were developed but only applied as retrospective analyses after build-up of resistance in the field. In this paper we focus on the evaluation of the inherent risk of the novel phenylpyrrole fungicide fludioxonil. We could demonstrate two biologically dissimilar resistance types: laboratory and field resistance. Fludioxonil laboratory resistant strains could be selected easily without the use of mutagens or UV irradiation. These spontaneously occurring laboratory resistant strains were consistently also resistant to dicarboximides. Classical genetic analysis showed that there is no cross resistance to fludioxonil in dicarboximide resistant field isolates. Reliability and pitfalls of the modified risk evaluation scheme of Gisi and Staehle-Csech (1988) are discussed.

INTRODUCTION

The idea of predicting the resistance risk of new candidate fungicides is not new. Dekker (1982) discussed the question "Can we estimate the fungicide resistance hazard in the field from laboratory and greenhouse tests?", Gisi and Staehle-Csech (1988) published a detailed protocol for the evaluation of the resistance risk of new fungicides and Brent *et al.* (1990) described the effort to combine genetic, monitoring, multifactorial and modelling approaches to develop the best judgement of risk before and during the early application of a fungicide. Although several authors have dealt with the theoretical aspects of the prediction of fungicide resistance the schemes which they proposed were only applied as retrospective analyses on existing resistance problems in the field. Currently several novel groups of fungicides such as phenylpyrroles, anilinopyrimidines and methoxyacrylates are in an advanced state of development and probably will enter the market within the next few years. More than twenty years of experience and a profound theoretical background in risk analysis can be used in the risk assessment concerning the new generation of fungicides. Active research has to be dedicated to the aspects: dynamics of fungal populations with respect to resistance, resistance risk assessment and resistance management. There is an ideal opportunity to apply our knowledge to predict the potential risk of resistance build-up and to develop and implement antiresistance strategies from the very beginning of the product's use in the field. We report on joint efforts and close cooperation in the risk assessment between industry, academia and registration authorities. In the present paper we give an overview of what has been done to evaluate the resistance risk of fludioxonil, a novel phenylpyrrole fungicide.

RESISTANCE RISK ASSESSMENT

As a whole the resistance risk is a combination of inherent resistance risk and management resistance risk (Staub and Sozzi, 1984). Management risk and inherent risk are equally important. However, in contrast to the inherent risk, the management risk can

be influenced by various means. "High risk" fungicides, when used with proper strategies (= low management risk), may not cause more overall risk than "low risk" fungicides used improperly (= high management risk) (Gisi and Staehle-Csech 1988). In this paper we focus on the evaluation of the fungicide - pathogen related inherent resistance risk.

Fungicide

Fludioxonil is a novel, non-systemic phenylpyrrole fungicide. It is a derivative of the antibiotic pyrrolnitrin and is highly active against a broad spectrum of fungi among Ascomycetes and Basidiomycetes. Fludioxonil is being developed for foliar use with *Botryotinia fuckeliana* as its major target pathogen (Gehmann 1990). Jespers (1994) demonstrated that the primary mode of action of this class of fungicides is new: it is based on the inhibition of transport associated phosphorylation of glucose.

Pathogen

B. fuckeliana (syn. *Botrytis cinerea*) causal agent of grey mould is an economically important pathogen on a wide range of host plants and it causes considerable damage during storage and transportation. *B. fuckeliana* grows fast on artificial and complex media and the sexual stage can be induced under laboratory conditions (Faretra *et al.* 1988) which makes this pathogen accessible to classical genetic analysis. It has also been successfully transformed making it available for molecular genetic studies (Hilber *et al.* 1994b). Beyond the genetic variability caused by mutation and sexual reproduction, *B. fuckeliana* additionally shows a genetic flexibility that may be caused by the selection of different alleles within the heterokaryon. High genetic variability and flexibility, high reproduction rate, wide host range and the possibility of saprophytic growth on virtually any plant debris favour a high inherent risk for resistance. In the past resistant *B. fuckeliana* populations were selected only few years after the introduction of the benzimidazoles and the dicarboximides.

DESIGN OF RESISTANCE RISK ASSESSMENT

Gisi and Staehle-Csech (1988) proposed a step by step procedure for estimating the resistance risk of new fungicides. Risk analysis, however, highly depends on the combination fungicide - pathogen and protocols can only be guidelines that have to be modified in each new case. In our evaluation of the inherent resistance risk of the combination *B. fuckeliana* - fludioxonil we adopted the protocol from Gisi and Staehle-Csech and modified it as shown in Figure 1.

Test Methods - Baseline sensitivities

To be able to test a new compound in standard agar plate assays at least two requirements have to be met: a) the fungicide must be soluble in a solvent that can be mixed with agar (and does not influence the pathogen) and b) the fungicide must be active in the *in vitro* test. Fludioxonil meets both requirements. The active ingredient (technical grade) was dissolved in ethanol and then added to the previously cooled agar. Mycelial growth tests and germination tests have revealed that fludioxonil is a highly active inhibitor of conidia germination and of mycelial growth. Since germination is inhibited 19 times less than mycelial growth, and evaluation of the germination tests is not always easy, we favoured the mycelial growth test as the *in vitro test*. EC_{50} values range from 0.08 to 0.2 mg/l for conidia germination and from 0.003 to 0.016 mg/l for mycelial growth. The variation in the sensitivities of isolates never exposed to fludioxonil (baseline sensitivities) was low and in the range of the variation known for dicarboximide fungicides.

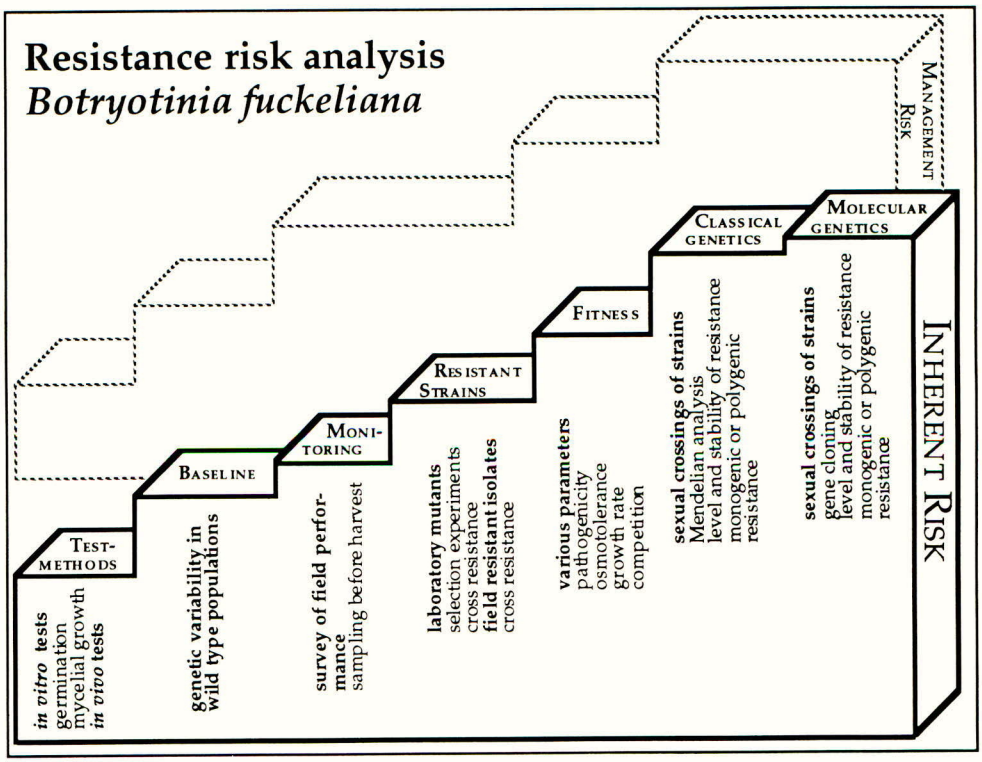
Monitoring

Fungicide resistance monitoring, testing the sensitivity of target organisms from field populations, is the basis not only of the management of fungicide resistance but also of the

resistance risk evaluation itself. As a part of the risk assessment monitoring has to start during product development, and before the start of sales. Companies should, however, prolong monitoring throughout the product's life span to be able to judge the success of their antiresistance strategies, and to review strategies as soon as necessary.

Results obtained with dicarboximides showed that resistant fungal strains occurred at a frequency of 1×10^{-7} before the first application of the novel ingredient (Martinetti 1986). In the first stage of monitoring it is important to monitor a large population. Changes in sensitivity to fludioxonil were intensively monitored by Ciba Geigy and by ourselves. Up to now more than 1000 isolates were tested. None of them showed stably reduced sensitivity to fludioxonil.

Figure 1: Modified Gisi and Staehle-Csech risk evaluation scheme



Resistant Strains

Early laboratory experiments with dicarboximides such as vinclozolin have shown that laboratory resistant strains could easily be selected by exposing conidia or mycelium to fungicide amended plates (Martinetti 1986, Schüepp *et al.* 1982), without mutagens or UV irradiation. Experiments in our laboratory have revealed that the same is true for fludioxonil (Hilber 1992, Hilber *et al.* 1993). Laboratory mutants that were selected in this way were cross resistant to both vinclozolin and fludioxonil. It did not matter whether these mutants were selected on vinclozolin or fludioxonil amended medium. "Training" experiments, involving repeated exposure to increasing, but sublethal concentrations of the fungicide did not yield resistant strains. We did not apply mutagens or UV radiation as

the frequency of spontaneously occurring laboratory mutants was very high. Such experiments would have needed the selection of mutants on fungicide amended media after the mutagenic treatment which would not have allowed the distinction between mutagen induced mutants and spontaneously occurring ones.

Dicarboximide resistant strains that were isolated from rotten grapes in the field showed a different resistance pattern. These field resistant strains revealed only a moderate level of resistance to vinclozolin while they were sensitive to fludioxonil. Laboratory resistant strains, in contrast, were resistant to fludioxonil and to vinclozolin, but this type of resistance was not encountered in the field.

Fitness

Field application of an active ingredient creates a specific selection pressure for the fungal population in addition to the various naturally occurring selection pressures due to adverse physical, chemical or biological factors. Under laboratory conditions only a limited number of fitness parameters can be investigated (Hilber *et al.* 1994a). In general laboratory resistant strains that were selected on either agar amended with vinclozolin or fludioxonil showed a highly decreased osmotic stress tolerance compared to their sensitive parental strains. These results were in accordance with data reported by Beever (1983).In parallel laboratory resistant strains lost their pathogenicity on apple cv. Golden Delicious. Competition experiments revealed similar results: laboratory resistant strains were not competitive they were suppressed by sensitive isolates after only few cycles of coculturing.

Dicarboximide field resistant strains behaved in the opposite way: although they showed a decreased sensitivity to vinclozolin their osmotic stress tolerance and their pathogenicity on apple was equal to that of sensitive strains. It must be assumed that the resistant strains sporadically occurring before the population had been in contact with the active ingredient can slowly improve their fitness under the long lasting selection pressure accompanying regular fungicide applications.

Genetic analysis

Classical genetics - Mendelian analysis

Mendelian analysis of sexual progenies of crosses between dicarboximide resistant field strains and sensitive strains, as well as of crosses between laboratory resistant and sensitive strains, revealed that resistance was due to mutation in one or two closely linked resistance genes. In the analysis of crosses we never found independent segregation of the dicarboximide and the phenylpyrrole resistance although field resistant strains only showed resistance to dicarboximides. Field and laboratory resistance are biologically dissimilar. This is of considerable importance in the judgement of the resistance risk of phenylpyrroles. Although knowledge about the difference between field and laboratory resistance has greatly increased we still do not know its genetic background. Classical genetic analysis is not sensitive enough to give the answer in this case. Therefore a molecular genetic approach is needed.

Molecular genetics

Molecular genetic techniques have improved dramatically over the last decade. PCR, a major breakthrough in this field, and its various applications are commonly used techniques in many laboratories. Compared to well documented model organisms such as *Neurospora* or *Saccharomyces*, little is known about the genetics of *B. fuckeliana*. Molecular genetic exploration of this pathogen was anticipated to be cumbersome but, reports on the segregation of DNA polymorphisms by RAPD analysis (Van der Vlugt-Bergmanns *et al.* 1993) and successful transformation of *B. fuckeliana* (Hilber *et al.* 1994b), have opened new perspectives. A molecular genetic approach could answer remaining questions which were left open by the less sensitive classical (Mendelian) approach. Molecular genetic analysis is a further tool to be used for resistance risk assessment.

CRITICAL EVALUATION

The novel botryticide fludioxonil shows similarities with the chemically unrelated dicarboximide fungicides. Leroux and coworkers (1991, 1992) hypothesised that dicarboximides and phenylpyrroles might have the same mode of action. First experimental data suggested that fludioxonil has a "high inherent risk". Experience with dicarboximides and benzimidazoles has taught us that resistant *B. fuckeliana* subpopulations can be selected very rapidly under heavy selection pressure. Laboratory data revealed that fludioxonil resistant strains can be selected easily without the use of mutagens or UV irradiation. These spontaneously occurring laboratory mutants are cross resistant to dicarboximides and fludioxonil.

Laboratory results were, however, completely contradictory to the performance in the field. Efficacy of fludioxonil was excellent in all our field trials. We could clearly demonstrate two biologically dissimilar resistance types: laboratory and field resistance. In plots where dicarboximide fungicide were applied only a few times in the past, we repeatedly found an increase in the resistance frequency from low levels to 100% after one to two application of dicarboximides (Hilber *et al.* 1994a). Fludioxonil, however, did not alter the dicarboximide resistance frequencies which was evidence for lack of cross resistance in the field.

Classical genetic analysis confirmed that there is no cross resistance to fludioxonil in dicarboximide field resistant strains. As this situation is neither matched by the term cross resistance nor by the term multiple resistance a new term has to be found. A final answer to Dekker's question "Can we estimate the fungicide resistance hazard in the field from laboratory and greenhouse tests?" cannot be given. The model of Gisi and Staehle-Csech is a good guideline for the experimental design but as demonstrated in our analysis it contains pitfalls. An exact calculation of a risk as it is suggested by the Gisi and Staehle-Csech model, is not feasible. The laboratory data presented in this paper have to be interpreted with care. No parallels to the phenomenon of the laboratory resistance was observed in the field. We assume that we have to pay attention to two significantly different processes: the selection for resistance and the selection for fitness. Under natural conditions in the field, even slight differences in fitness with regard to various, not yet elucidated aspects can essentially improve the proliferation potential or the survival mechanisms of a specific strain. As demonstrated in laboratory conditions, selection for resistance is fast and constitutes, no doubt, a potential risk. Selection for fitness in resistant strains, however, seems to be very slow. This may explain why the build-up of a resistant fungal population having acquired normal fitness and thus being competitive with sensitive strains usually takes years. With the current state of knowledge we assume that the inherent risk of fludioxonil is medium. However, the management resistance risk is high as there are no fungicides available yet that are suitable partners. The limitation of applications to one or two sprays per season is difficult to be enforced. In the case of dicarboximides this strategy was not effective as demonstrated in field experiments (Hilber *et al.* 1994a). Industry and regulatory authorities have the obligation to enforce "true" resistance strategies which could be a mixture of phenylpyrroles and anilinopyrimidines. Compounds of both classes are in the final steps of registration. Management risk could be kept low resulting in a good chance of success in *Botrytis* management.

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