Comparative studies on genetic variability and fungicide resistance in *Botryotinia fuckeliana* (de Bary) Whetzel against vinclozolin and the phenylpyrrole CGA 173506

Inauguraldissertation
zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch- Naturwissenschaftlichen Fakultät
der Universität Basel

von

Urs Werner Hilber
aus Degersheim- Magdenau SG

Basel, 1992

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Genehmigt von der Philosophisch- Naturwissenschaftlichen Fakultät
auf Antrag der
Herren Professoren Dr. F.J. Schwinn und Dr. T. Boller

Basel, den 28. April 1992

Prof. Dr. J.-M. Le Tensorer
(Dekan)

Dedicated to
Maja Bodmer

Contents

Preface

The following studies were realized at the Swiss Federal Research Station at Wädenswil from 1989 to 1992.

I like to thank all who helped me with their contributions to achieve the results presented in my PhD thesis. I am grateful to my teachers Prof. Dr. F.J. Schwinn and PD Dr. H. Schüepp who gave me the exceedingly interesting topic and helped me at all times through their valuable scientific discussions. I am very grateful to PD Dr. H. Schüepp for promoting me at the Research Station and giving me the chance to cooperate with our guest scientists, Yufen Zhang from China and Prof. Dr. Robert N. Goodman from USA. I further thank Prof. Dr. Th. Boller for his mandate as a referee.

Plant protection and fungicide resistance are topics of high socioeconomic interests. The present project was most interesting as the constellation of a cooperation of academia (University of Basle), national authorities (Swiss Federal Research Station) and industry (Ciba-Geigy Ltd.) gave promise of a well balanced approach. In this context I express my thanks to Ciba-Geigy Ltd., Agricultural Division, for financial support and to its representatives Dr. Th. Staub, Dr. P. Urech, Dr. D. Sozzi and Dr. C. Nuninger for helpful discussion of the project, and to the Director of the Swiss Federal Research Station at Wädenswil, Dr. W. Müller and his staff of administration for their hospitality.

I thank, W. Siegfried and Dr. E. Bosshard who were excellent teachers in the field and our court photographer, K. Vogelsanger, for technical support. For making the SEM available to me and for technical assistance, I thank Prof. Dr. H. Hohl and U. Jauch from the University of Zürich. I also appreciate the help from my close friend Dr. M^{c}Virus (Dr. Th. Hasler) whose useful advice helped me solve many computer problems. M. Buraglio and all the staff and apprentices of the department of plant pathology, I thank for having made me feel at home in this department.

I highly appreciated the friendship and the open, happy characters of B. Buchmann and K. Langenegger who both assisted me in experiments and gave the psychological support which I found essential when working with *Botryotinia fuckeliana* ! For all their contributions I would like to express my special thanks.

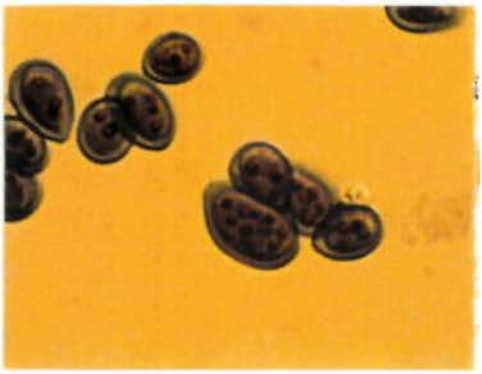
I further would like to thank all my friends and my parents for their great forbearance during my temporary withdrawal from normal life and Jeff Smith for careful reading of this manuscript and for his adjustments to the grammar.

Last but not least I thank my girl friend Maja Bodmer to whom this thesis is dedicated. As she is an expert in *Botryotinia fuckeliana* our, sometimes contradictory, discussions were not always settled at the end of work time. I thank her for all her efforts, the extra hours, her tremendous interest in my work and for all our fruitful discussions.

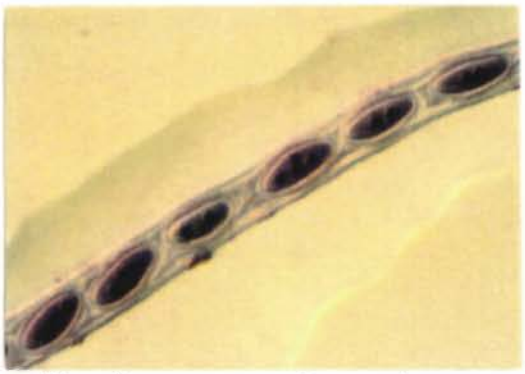
Disease cycle of Botryotinia fuckeliana (de Bary) Whetzel

ascus and liberated ascospores

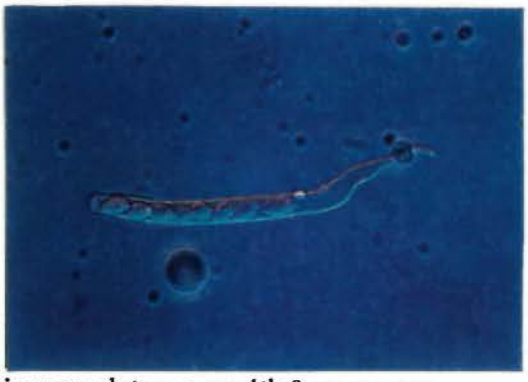
single ascospore cultures



multinuclear conidia^



multinuclear ascospores in ascus^

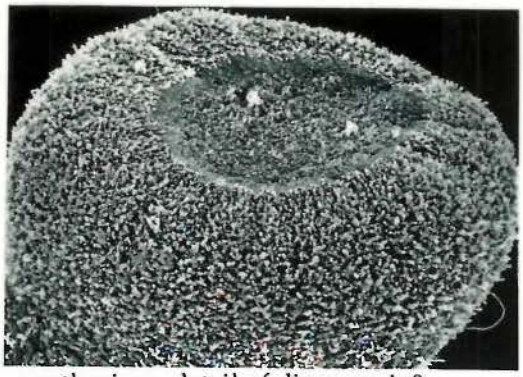


inoperculate ascus with 8 ascospores

sclerotia and apothecia of Botryotinia fuckeliana

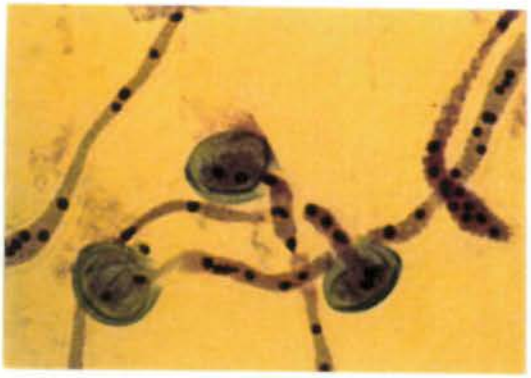
ana
fuc

apothecium, detail of disc centre°

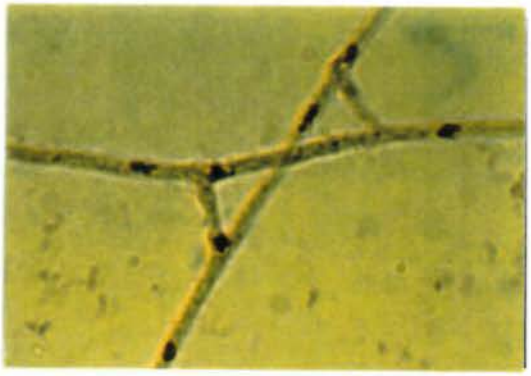


apothecium, detail of disc margin°

develop



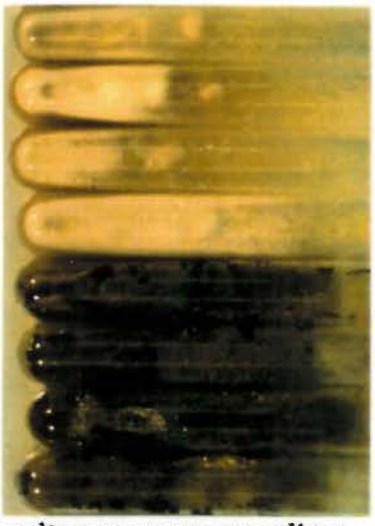
multinuclear germ tubes^



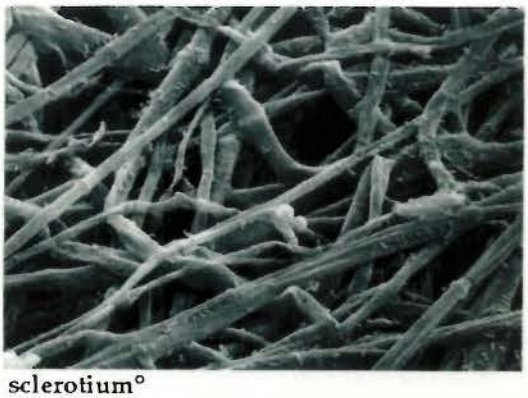
anastomoses, exchange of nuclei^

norph of Botryotinia
eliana (Botrytis cinerea)

conidiophore

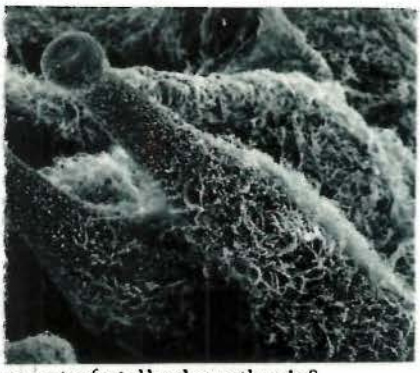


culture on pea agar medium

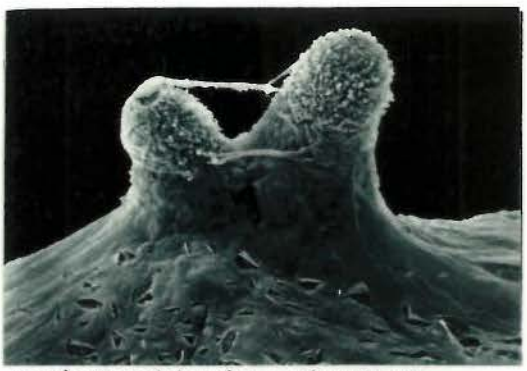


sclerotium°

microconidia^



ment of stalked apothecia°

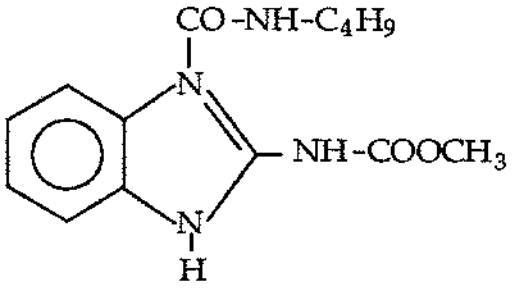


apothecia arising from sclerotium°

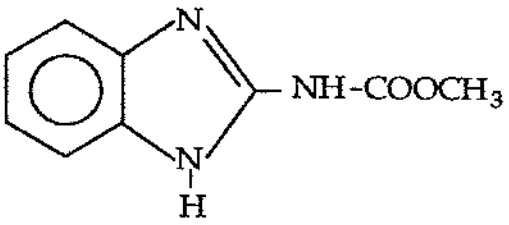
^HCl Giemsa stain
° SEM photographs technical assistance: U. Jauch

Structure formulae of compounds used in these studies

Methylbenzimidazolcarbamate fungicides

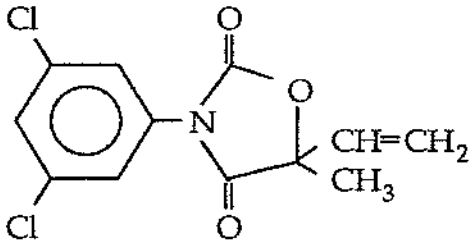


benomyl

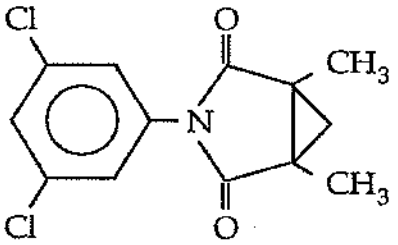


carbendazim

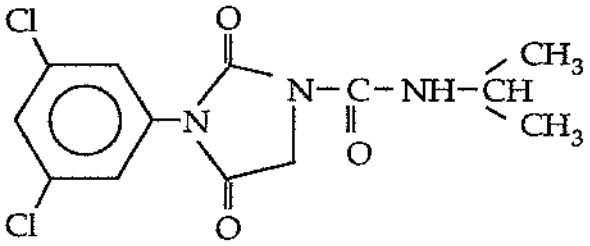
Dicarboximide fungicides



vinclozolin

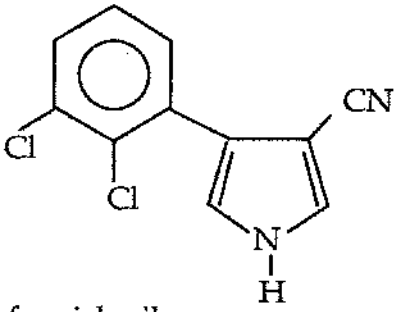


procymidone

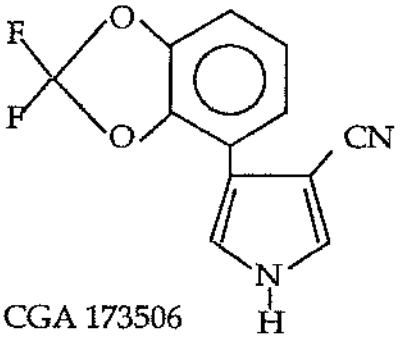


iprodione

Phenylpyrrole fungicides



fenpiclonil



CGA 173506

General Introduction

The genus *Botrytis* (βοτρυς = a bunch of grapes) was established by Micheli in 1729. It was validated by Persoon in 1801 and revalidated by Nocca and Balbis in 1821 and by Fries in 1832 (Gregory, 1949, Coley-Smith, 1980). Although Fries described *Botrytis cinerea* under the rules of botanical nomenclature, Persoon's description of *B. cinerea* is generally referred to (Groves and Loveland, 1953).

In 1866, de Bary reported for the first time *Peziza fuckeliana* to be the teleomorph of *B. cinerea*. Three years later Fuckel established the new genus *Sclerotinia* with *Sclerotinia fuckeliana* the second species mentioned (Gregory 1949). The connection of *B. cinerea* and *P. fuckeliana*, or *S. fuckeliana*, respectively, was generally accepted until the beginning of this century but was then questioned because de Bary's specimens and descriptions were lost. The situation was resolved, when in 1949, Gregory discovered de Bary's type specimens in the British Museum of Natural History in London. He found a slide with sections of apothecia labelled *P. fuckeliana* in de Bary's handwriting. Whetzel established the genus *Botryotinia* and described the new combination *Botryotinia fuckeliana*. Details on the history of the description of *B. fuckeliana* are summerized by Gregory (1949).

Groves and Drayton (1939) for the first time reported the production of apothecia of *B. fuckeliana* under semi-controlled conditions. Besides this report, there are only a few other reports published concerning the occurrence of *B. fuckeliana in vitro* (Groves and Loveland, 1953, Lorenz and Eichhorn, 1983) or in the field (Kublitzkaya and Rjabtzeva, 1968, 1970, Polach and Abawi, 1974, 1975). Faretra and Antonacci (1987), Faretra and Pollastro (1988) and Faretra et al. (1988) investigated the production of apothecia of *B. fuckeliana* under fully controlled environmental conditions. They developed a reliable method for the production of apothecia *in vitro* which was also successfully used in the present study. The genetics of *B. fuckeliana* were hardly investigated due to the difficulties of obtaining apothecia (Grindle, 1983). Even though the procedure still remains laborious and time consuming, apothecia can now be readily induced *in vitro* and the sexually produced ascospores are accessible for genetic analysis.

B. fuckeliana can infect an extremely wide range of host plants including field and glasshouse vegetables, fruits, ornamentals, bulb and corn producing monocotyledons and forest tree seedlings. In addition this fungus can cause considerable damage to plants as well as plant products during storage and transportation (Coley-Smith, 1980, Jones and Sutton, 1984, Rosenberger, 1990, Bulit and Dubos, 1988, Snowdon, 1990, 1991).

Grey mould is the most widespread and important disease of grapes, occurring wherever the crop is grown (Snowdon, 1990). Quality as well as quantity of grapes can be drastically reduced by *Botryotinia* bunch rot. Modified chemical composition in grape berries, conversion of simple sugars (glucose and fructose) to glycerol and gluconic acid as well as the production of enzymes catalyzing the oxidation of phenolic compounds are due to the infection of *B. fuckeliana*; this may lead to serious qualitative damage in vine production (Bulit and Dubos, 1988). Vines produced from rotten grapes have off flavors and are sensitive to oxidation and bacterial contamination which makes them unsuitable for aging. Even though in most cases *B. fuckeliana* causes severe damage it can be beneficial when occurring shortly before harvest, causing a condition termed noble rot. Due to damage of the cuticle by *B. fuckeliana*, water evaporates from the grape berries. As a result, the concentration of sugar rises which leads to heavy sweet vines, as the Tokays of Hungary, the Sauternes of France and Trockenbeerenauslese of Germany.

Chemical control of plant diseases and occurrence of resistance problems are of a special socioeconomic

importance (Schwinn, 1982, Köller and Scheinpflug, 1987). The first highlights in fungicide development were the introductions of Bordeaux mixture (1855) and organomercurials (1913) (Eckert, 1988). These fungicides were non selective and provided environmental problems due to the input and accumulation of heavy metals in the ground. In the Eastern part of Switzerland, between World War I and II, the input of copper was up to 80kg per ha and year (Räz et al., 1987) whereas it is today, due to modern chemical control measures, 3 to 4kg per ha and year (Anonymous, 1991). Until the early 60's the inorganic compounds were partly replaced by synthetic, protective, multisite inhibitors (Eckert, 1988), mainly dithiocarbamates and phthalimides. Although these compounds were widely and exclusively used, the development of resistance was not encountered (Köller and Scheinpflug, 1987). From the late 60's onwards, starting with the introduction of the methyl-benzimidazolecarbamate fungicides (see p. 3), selective single- site inhibitors came on the market. These and other systemic, highly selective fungicides like the phenylamides, the SBI's (Sterol Biosynthesis Inhibitors) as well as the non- systemic dicarboximides show a very positive ecological and toxicological behavior. Their high selectivity, however, contains the risk of facile development of resistance (see chapter 3). This is demonstrated by the accelerated increase in the number of resistant fungal genera since their market introduction. In most cases the development of resistance can be explained by the selection of spontaneous mutants in the pathogen population that have a decreased biochemical target affinity. Most cases of rapid development of practical resistance could be ascribed to mutants possessing a single major gene for resistance (Eckert, 1988). In one step resistance, mutation of a single major gene is all that is required for the fungus to acquire the highest resistance possible (Georgopoulos, 1988). In the case of SBI's, however, resistance is of polygenic nature, i.e., it results from the additive effects of several minor genes for resistance (Köller and Scheinpflug, 1987, Georgopoulos, 1988). Practical resistance in the field does therefore not develop as quickly as in the previously mentioned cases; for example, powdery mildew of small grain cereals is only slowly shifting towards reduced sensitivity (Schwinn and Morton, 1990). However, in both cases, of one step or multistep resistance the genetic potential for overcoming the fungicide toxicity is present and the resistant biotype is nurtured by a selective environment that favors its multiplication over that of other components of the population (Georgopoulos, 1987, Eckert, 1988). With major gene control of resistance, the sensitivity distribution of the target population is discontinuous and the response to selection is qualitative. In polygenic resistance the overlapping of genotypic classes results in a continuous, unimodal frequency distribution. The observed change is a gradual, quantitative shifting of the whole distribution toward reduced sensitivity (Georgopoulos, 1988).

Before the introduction of methyl-benzimidazolecarbamates, no highly effective means of control were available for grey mould. Substantial improvement was obtained by their introduction in the late 60s and again by the introduction of the dicarboximides (see p. 3) in the early 70s. In both cases, however, few years after wide and exclusive practical application of these chemicals, buildup of resistance was encountered (Schüepp and Lauber, 1977, Holz, 1979). The buildup of resistance to methyl-benzimidazolecarbamates in *B. fuckeliana* in Swiss vineyards provided a classic example of how such resistance appeared and persisted (Staub, 1991). The stability of resistance in *B. fuckeliana* was shown by Schüepp and Küng (1981) who compared the frequency of resistant *B. fuckeliana* isolates from Switzerland, collected in 1974 when the use of methyl-benzimidazolecarbamate fungicides was discontinued, with that of resistant strains collected in 1978. Even though the selection pressure was absent for four years the proportion of resistant strains persisted between 50 to 60%. In contrast, in the case of the dicarboximides the frequency of resistant strains decreased after stopping treatments (Schüepp and

Siegfried, 1983, Martinetti, 1986). They did, however, not disappear totally (Löcher et al., 1987).

Under these circumstances the phenylpyrrole compound CGA 173506, presented by Gehmann et al. (1990), is a highly welcome novel active ingredient to control *B. fuckeliana*. To ensure its long-term use in practice, the early evaluation of the risk of resistance, and the development of a suitable antiresistance strategy are of prime importance. The latter may consist of alternating spray programs or prepacked mixtures (DeWaard, 1988) whereby today mixtures are prevailing (Schwinn and Morton, 1990, Staub, 1991).

The use of dicarboximides as companion partners in mixtures with CGA 173506 depends on their resistance risk. In this context the population dynamics of *B. fuckeliana* field resistant to dicarboximides was studied in a vineyard. The influence of one to two dicarboximide applications per season as well as the effect of one year free of applications was investigated in a population of *B. fuckeliana* on the low susceptible grape-vine variety Blauburgunder, clone Mariafelder; for details see **chapter 1.**

Hansen and Smith (1932) and Menzinger (1965) described the formation of anastomoses and the exchange of nuclei between different hyphae in *B. fuckeliana*. Menzinger (1965) proved that numerous monoconidial isolates of *Botryotinia* were heterokaryons and, only in one case, did he succeed in producing morphologically recognizable heterokaryons from homokaryons.

Beyond the genetic variability which is, in common for all living organisms, caused by mutation and sexual reproduction *B. fuckeliana* shows additional genetic flexibility caused by the selection of different alleles within the heterokaryon, a characteristic feature of multinucleate (heterokaryotic) organisms.

Due to the spontaneous occurrence of mutations towards fungicide resistance and the possible occurrence of nuclei carrying alleles coding for sensitivity or for resistance in the same heterokaryon, alleles for resistance towards a novel fungicide can easily be selected when selection pressure exerted by this novel fungicide is applied. Leroux et al. (1977), Schüepp and Küng (1978) and Pommer and Lorenz (1982) reported the ease of selecting dicarboximide laboratory resistant strains of *B. fuckeliana* without using UV irradiation or other mutagenic agents.

The occurrence of laboratory resistant strains does, however, not necessarily imply the buildup of resistance in the field (Dekker, 1982a). The relative fitness of resistant strains compared to sensitive strains in the field is of paramount importance for the development of resistant populations (Dekker, 1982a). Acquisition of resistance is often linked with loss or decrease of fitness (Dekker, 1982a, Brent et al., 1990). For the assessment of a resistance risk it is thus essential to study the fitness of resistant and sensitive strains. Although in the laboratory only a limited number of fitness parameters (e.g., growth rate, sporulation, osmotolerance, competitive ability, pathogenicity and virulence) can be studied, which reflects a limited view of the much more complex situation in the field, studies on the fitness of resistant strains (compared to sensitive strains) provide an important tool in the evaluation of the resistance risk. In the absence of selection pressure, strains of *B. fuckeliana* that are resistant to methyl-benzimidazolecarbamates typically are as fit as sensitive strains which leads to a stable proportion of resistant strains in the field. However, strains that are resistant to dicarboximides, show a decreased fitness indicated by a decreasing proportion of resistant strains in the field in the absence of selection pressure. In this study the effect of the acquisition of laboratory resistance to CGA 173506 in *B. fuckeliana* on fitness parameters was studied with regard to the significance for the buildup of a resistant population in the field **(chapters 3 and 4).**

Good fungicide resistance monitoring (= testing for sensitivity of target organisms in field populations) is a cornerstone of the management of fungicide resistance (Brent, 1988). As the detection of resistant

strains is based on the comparison with data obtained from sensitive isolates, the evaluation of base-line sensitivity (= the genuine sensitivity of untreated wild type isolates) is crucial (Georgopoulos, 1982, Georgopoulos and Dekker, 1982, Brent, 1988 and Brent et al. 1990). Isolates of *B. fuckeliana* from strawberries that never received any fungicidal treatments in the field were collected at two locations. In the second chapter of this investigation the base- line sensitivity of CGA 173506 was established for these isolates.

Because the sexual stage of *B. fuckeliana* rarely occurs, genetics of fungicide resistance have not been investigated fully in this pathogen. However, recent work by Faretra and Pollastro (1991), who managed to obtain sexual reproduction under laboratory conditions, has clarified the genetic basis of resistance against vinclozolin and methyl-benzimidazolecarbamates. Resistance to both fungicides is each encoded by a single polymorphic major gene. Faretra and Pollastro (1991) further recognized three classes of alleles at each locus. Based on the methods of Faretra and Pollastro (1988) the inheritance patterns of resistance against CGA 173506 and against vinclozolin and of related fitness parameters in *B. fuckeliana* were investigated (**chapter 4**). In contrast to Faretra and Pollastro (1991) who were using random spore analysis, the genetic basis of resistance and pleiotropic effects on fitness characters were studied using classical tetrad analysis.

In summary, the goal of the present investigations was to study genetic variability in *B. fuckeliana* by comparing fungicide resistance to vinclozolin and the phenylpyrrole CGA 173506 and to assess the resistance risk of the novel compound CGA 173506. In a field study (**chapter 1**), the occurrence of vinclozolin field resistance under the influence of restricted use of dicarboximides in a weakly susceptible grape variety was studied. Population dynamics of *B. fuckeliana* resistant to dicarboximides were evaluated during 3 successive seasons from 1989 to 1991. In **chapter 2** the effect of CGA 173506 and of vinclozolin on the pathogen was compared *in vitro* and base-line sensitivity concerning both active ingredients of two wild type populations was evaluated. In **chapter 3** the potential of the fungus to develop laboratory resistance against CGA 173506 and against vinclozolin was studied. Morphological characters and fitness parameters of selected mutants were compared. The genetic basis of resistance against CGA 173506 and against vinclozolin was studied by using sexual progenies of *B. fuckeliana* (**chapter 4**). Inheritance of fitness parameters affected by the acquisition of resistance to vinclozolin or CGA 173506 was studied by tetrad analysis (**chapter 4**).

1. Field study on the population dynamics of *Botryotinia fuckeliana* (de Bary) Whetzel resistant to dicarboximides

1.1 Introduction

Iprodione and vinclozolin were first introduced in Switzerland in 1976 for large field trials and were later registered in 1977 (Schüepp, 1977). Due to good performance they were intensively used to control *B. fuckeliana* (grey mould); 4 to 5 treatments per season were allowed in grape-vines (Schüepp, 1977, Beetz and Löcher, 1979). Leroux et al. (1977) and Schüepp and Küng (1978) could easily select highly resistant strains of *B. fuckeliana* in the laboratory. During the intensive monitoring program which commenced in 1976, the first low level field resistant strains were found in 1979, three years after the first use of the dicarboximides in Switzerland. Holz (1979) reported for the first time loss of efficacy of the dicarboximides in the Mosel valley in Germany. In the vineyards, monitored by Schüepp and Siegfried (1983), the percentage of resistant strains increased drastically from about 40% in 1979 to almost 100% in 1982 but a decreased efficacy only was found from 1982 onwards. Whereas Schüepp and Siegfried (1983) no longer advised the use of dicarboximides in Swiss vineyards where field resistance occurred, other authors recommended a limitation to two, in exceptional cases three, applications per season (Gouot, 1988, Löcher et al. 1985, Löcher et al. 1987, Löcher, 1988, Wade, 1988). In contrast to the resistance to methylbenzimidazoles which was stablely maintained in the population in the absence of a selection pressure of the fungicide, the resistance to dicarboximides was not stable. Although the resistant population did not disappear completely, even after several years without a selection pressure (Martinetti, 1986, Löcher et al. 1987), the proportion of resistant strains in the pathogen population decreased (Wade, 1988).

After the occurrence of field resistance, many scientists started to study the population dynamics of *B. fuckeliana* resistant to dicarboximides (Leroux and Clerjeau, 1985, Martinetti, 1986, Löcher et al. 1987, Pak et al. 1990). They mainly investigated seasonal changes in the frequency of resistant strains of *B. fuckeliana* in the population on susceptible grape varieties in intensively treated vineyards. The influence of reduced selection pressure on the development of the resistant population has not been investigated to date. Delp (1980) and Staub and Sozzi (1984) assumed that a reduction of the number of treatments would prevent or at least delay the buildup of resistance. In this context, the changes in sensitivity of *B. fuckeliana* to dicarboximides were monitored over three years under practical conditions in the vineyard in Walenstadt, Switzerland, where dicarboximides were applied at the most 2 times per season. During this investigation only one treatment at early bunch closure was applied in the first year and no treatment was applied in the second year. In the third year one third of the vineyard remained untreated whereas the other two thirds were treated twice with vinclozolin.

Due to the low susceptibility of the grape variety Blauburgunder, clone Mariafelder, the disease pressure in the vineyard in Walenstadt, Switzerland, has been low. Dicarboximides were therefore only sprayed 7 times since the beginning of the introduction of this group of fungicides (Table 1.1) and before these studies were started.

1.2 Materials and Methods

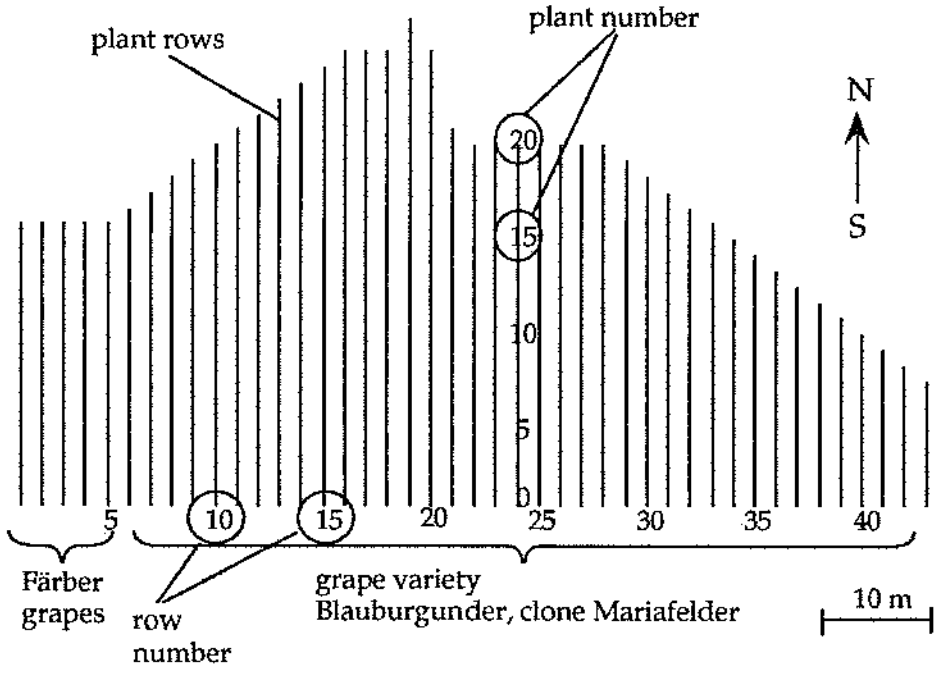
The field trials were carried out in the upper plane of the vineyard in Walenstadt which consists of 39 rows with up to 30 plants of Blauburgunder clone Mariafelder per row (Fig. 1.1). This plot totally re-

ceived 10 treatments with dicarboximides from 1983 to 1991 (Table 1.1,2). During the vegetation periods of 1989 to 1991 varied treatments of dicarboximides were applied (Table 1.2). Iprodione or vinclozolin, respectively, (Maag, AG; 1kg a.i. per ha) were sprayed with a high pressure (2MPa) main sprayer connected to a tube with two nozzles of 1.2mm width using a water rate of 1500 to 2000L/ha. In 1989 iprodione was applied at early bunch closure to the entire plot. During 1990 no dicarboximides were used at all. In 1991 rows 5 to 17 were treated with vinclozolin twice, at early bunch closure and at veraison, with an air blast sprayer using 400L/ha whereas rows 18 to 43 remained untreated. The proportion of isolates of *B. fuckeliana*, resistant to dicarboximides was monitored at early bunch closing, prior to treatment, and before harvest. Control of pests and pathogens other than *B. fuckeliana* was carried out following the Swiss plant protection recommendations for vine growing (Anonymous, 1991).

The detection limit of resistant strains in a fungicide resistance monitoring program directly depends on the number of samples (isolates), e.g., the minimum detection limit with ten isolates is 10% (1 resistant isolate out of 10) whereas it is 5% with 20 isolates (1 resistant isolate out of 20) and 1% with 100 isolates (1 resistant isolate out of 100) (Leroux and Clerjeau, 1985). For dicarboximide resistance monitoring a detection limit of 5%, that is a sample size of 20 isolates was feasible.

Freshly sporulating isolates of *B. fuckeliana* (19 to 25) were randomly collected within the rows 6 and 26 from leaves, pea sized berries, and mature berries, respectively. Conidia were transferred from sporulating colonies to malt agar (12g agar-agar, Merck and 15g malt extract, Difco) amended with 100mg streptomycinsulfat/L and 100mg oxytetracyclin/L. After an incubation period of 3 days at 20°C in the dark, the colonies were transferred to pea agar (15g agar-agar, Merck, 168g green peas and 5g sac-

Fig. 1.1: Vineyard in Walenstadt, Switzerland



charose per liter, pH=6) and incubated at 20°C under fluorescent and black light to enhance sporulation. Spore suspensions of 10^5conidia/ml were prepared from intensively sporulating cultures. 50µl of the suspensions were added onto malt agar discs of a diameter of 10mm. The discs were incubated for 17h at 20°C in the dark and then placed upside down onto malt agar plates amended with 1, 3 and, in 1990 and 1991, with 10mg vinclozolin/L. Mycelial growth was assessed by measuring the mean colony diameter after an incubation period of 3d at 20°C in the dark. Mycelial growth on agar amended with fungicide was compared to mycelial growth on fungicide free agar (check). Isolates which were able to grow on agar amended with 1, 3 or 10mg vinclozolin/L more than 50% of the diameter of the control were classified as resistant at a level of 1, 3 or 10mg vinclozolin/L, respectively. All experiments were replicated threefold.

1.3 Results

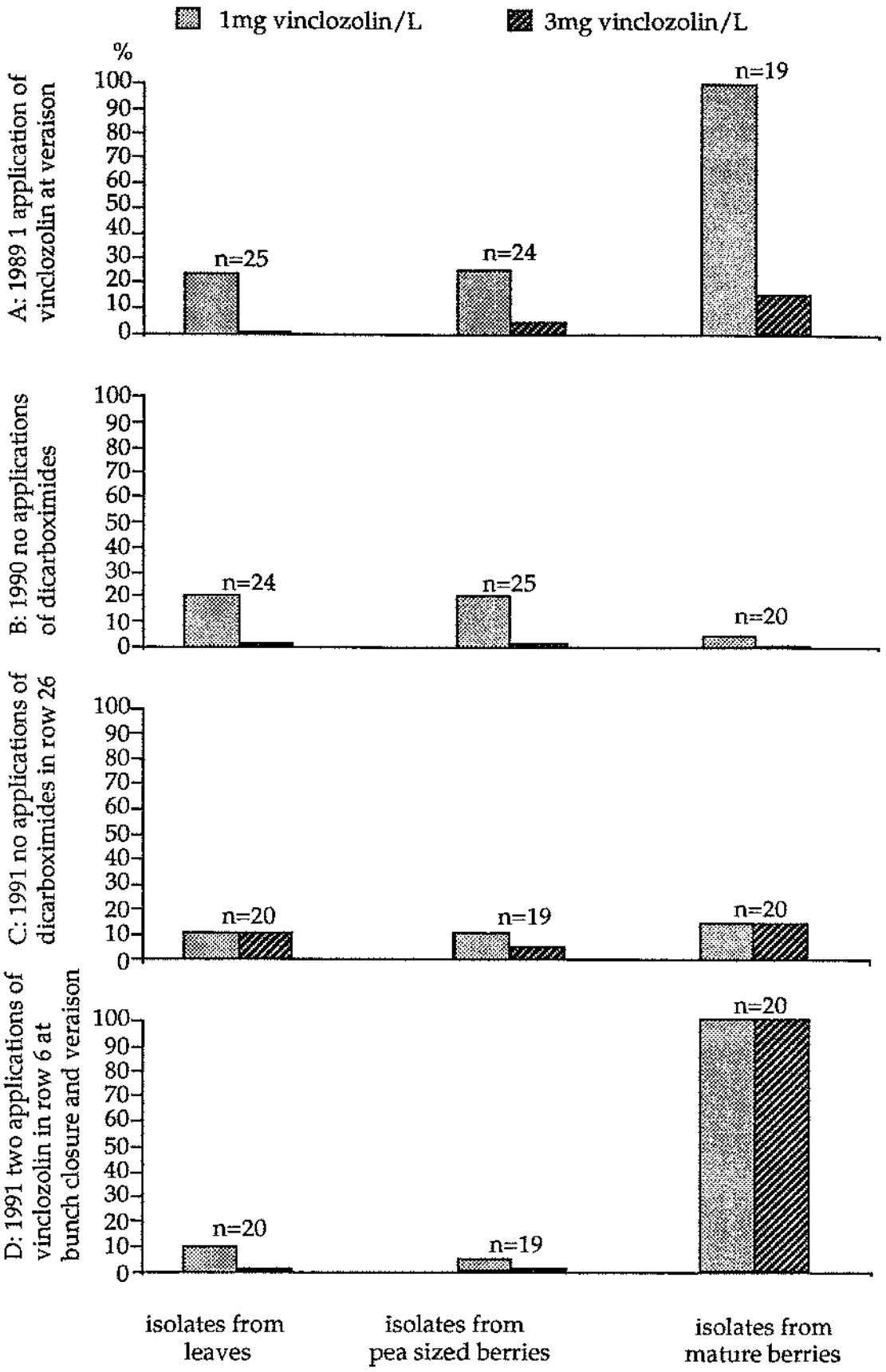
Table 1.2 shows the dates of dicarboximide applications and dates of isolations of *B. fuckeliana* from 1989 to 1991. Before the dicarboximide treatments the abundance of dicarboximide field resistant strains was low; as shown in figure 1.2A in 1989 prior to the field treatment with iprodione 24% of the isolates from grape leaves and 25% of pea sized berries were resistant at a level of 1mg vinclozolin/L. Isolates from leaves being resistant at a level of 3mg vinclozolin/L were not detectable, whereas 5% of the isolates from pea sized berries were resistant at this level. After the treatments the abundance of dicarboximide field resistant strains was high; all the isolates taken from mature berries after treatment were resistant at a level of 1mg vinclozolin/L and 16% also were resistant at a level of 3mg vinclozolin/L. There was no significant difference in the per cent of resistant isolates from leaves and pea sized berries, neither for a level of 1 nor of 3mg vinclozolin/L. The difference in the proportion of resistant isolates from leaves, pea sized berries and mature berries was highly significant (Contingency table analysis, p=0.0001).

In 1990 no dicarboximides were applied at all. None of the isolates from leaves, pea sized and mature berries was resistant at a level of 3mg vinclozolin/L (Fig. 1.2B). On malt agar amended with 1mg vinclozolin/L 21% of the isolates from leaves, 20% of the isolates from pea sized berries and 5% of the isolates from mature berries were resistant, the differences were not significant (Fig. 1.2B). From this it was concluded that the increase in the frequency of strains that were resistant at a level of 1 and 3mg vinclozolin/L in 1989 was due to the one application of iprodione before bunch closure.

Table 1.1: Applications of dicarboximides from 1983 to 1988 in the vineyard in Walenstadt

year	number of applications	date of application	fungicide
1983	0	---	---
1984	1	21. August	iprodione
1985	1	19. August	procymidone
1986	2	22. July	procymidone
		21. August	iprodione
1987	2	5. August	iprodione
		1. September	iprodione
1988	1	28. July	iprodione

Fig. 1.2: Percent of field isolates growing ≥ 50% of control on agar amended with 1 and 3 mg vinclozolin/L



In 1991 *B. fuckeliana* was isolated from rows 6 and 26 (Fig. 1.1). Row 6 was treated before bunch closure and at veraison whereas row 26 remained untreated (Table 1.2). Figures 1.2C and D show that none of the isolates taken from leaves and pea sized berries of row 6 were resistant at a level of 3mg vinclozolin/L whereas 10% isolated from leaves and 5% isolated from pea sized berries from row 26 did show resistance. The differences between leaves and pea sized berries and between row 6 and 26 prior to treatment were not significant, neither for the level of 1 nor of 3mg vinclozolin/L. 15% of the isolates from mature berries of the untreated row 26 were resistant at a level of 1 and of 3mg vinclozolin/L (Fig. 1.2C). There was no significant increase in the per cent of resistant strains between leaves, pea sized berries and mature berries in the untreated row 26 (Fig. 1.2C), whereas there was a significant increase (contingency table analysis p= 0.0001) in the treated row 6 (Fig. 1.2C). All the isolates from mature berries of row 6 were resistant at a level of 1mg vinclozolin/L and 3mg vinclozolin/L.

From 1989 to 1991 isolates that were resistant at the level of 10mg vinclozolin/L were not found, their frequency was thus lower than 5%.

1.4 Discussion

Although the buildup of populations of *B. fuckeliana* resistant to dicarboximides occurred soon after the intensive use of these fungicides (Holz, 1979, Beever and Brien, 1983, Schüepp and Siegfried, 1983, Northover and Matteoni, 1986) the occurrence of resistant strains was not always positively correlated with loss of efficacy (Schüepp et al. 1982, Pommer and Lorenz, 1982, Northover, 1988). Beever et al. (1991) suggested the frequency of resistance genes to

Table 1.2: Monitoring from 1989 to 1991 in Walenstadt ; evaluation of seasonal shifts in dicarboximide sensitivity prior and after treatments with dicarboximides

date of sampling	sampled part of plant	date of treatment	application stage
6.7.89	leaves		
6.7.89	young berries		
28.9.89	mature berries	16.8.89[a]	veraison
12.7.90	leaves		
12.7.90	young berries		
15.10.90	mature berries		
26.6.91	leaves		
18.7.91	young berries		
		24.7. 91	bunch closure
		20.8. 91	veraison
26.9.91	mature berries[b]		
26.9.91	mature berries[c]		

[a] all rows treated with Rovral (iprodione)

[b] from row 6, treated with Ronilan (vinclozolin)

[c] from row 26, untreated

be the result of the interplay between selection for these genes determined by fungicide application, and selection against these genes due to their effects on fitness. Resensibilisation of a resistant population to a high extent due to reduced fitness in resistant strains is still not fully understood. Results from laboratory experiments did not always reflect the situation in the field as it will also be demonstrated in chapter 3. Under laboratory conditions only a limited number of fitness parameters can be investigated whereas in nature the number of parameters affecting fitness is enormous. This might be a possible explanation for the partly contradictory results from field and laboratory studies.

Northover (1988) showed in one vineyard a decrease of the resistance frequency from September to July whereas in three other vineyards he found no significant difference in the resistance frequency in September and in July. Löcher et al. (1987) concluded from a declining proportion of resistant strains from harvest to the first application of dicarboximides to treat as late as possible in the growing season. They showed two properly timed treatments to be as effective as four treatments. Leroux and Clerjeau (1985) reported similarities in the population dynamics between vineyards in the Champagne and the Alsace where they found high resistance frequencies at harvest and in the following spring. They found thus a different behavior in the Loire Valley where they observed a decrease in resistance frequency during this period, which they attributed to a dilution effect of sensitive inoculum from surrounding vineyards. Pak et al. (1990), however, by concluding from their own results, attributed these findings of Leroux and Clerjeau (1985) to dynamics within the vineyards themselves.

In the intensively treated vineyard in Wädenswil, Martinetti (1986) applied 4 treatments of vinclozolin which resulted in 100% resistant strains. The present study, however, even showed that in the restrictively treated vineyard in Walenstadt, during 1989 one single application of iprodione and during 1991 two applications of vinclozolin, were also sufficient for the resistant population to reach a level of 100%. In agreement with Löcher et al. (1985), our results from harvest 1989 and early bunch closure 1990 indicate a decrease in resistance frequency from 100% to at most 21% for strains of a level of 1mg vinclozolin/L (Fig. 1.2). In the absence of a selection pressure in 1990 and in the untreated rows also in 1991 resistant strains at the level of 1mg vinclozolin/L did not disappear.

Delp (1980) and Staub and Sozzi (1984) suggested that the development of fungicide resistance can be prevented or retarded by limiting the number of treatments in order to reduce the selection pressure and by combining or alternating active ingredients with different modes of action. The present investigation in the vineyard at Walenstadt clearly demonstrated that the restriction in the number of applications of dicarboximides to at most two treatments per season, from the very beginning of the commercial introduction of the dicarboximides as well as the use of less susceptible grape varieties as Blauburgunder, clone Mariafelder did not prevent the buildup of a resistant population. These results complemented those of Löcher et al. (1985), Leroux and Clerjeau (1985), Martinetti (1986) and Pak et al. (1990) who investigated the seasonal fluctuations in resistance frequency in vineyards that were intensively treated with dicarboximides for several years. Even though the population of *B. fuckeliana* was small, due to the reduced susceptibility of the chosen grape variety and the selection pressure by only one or two fungicide applications was low, the population dynamics observed during the three years field study were comparable to those described by the aforementioned authors.

This study showed that within the small population of *B. fuckeliana* in the vineyard at Walenstadt, a resistant subpopulation did build up despite cautious use of dicarboximides. In this context, the novel phenylpyrrole fungicide CGA 173506, might provide an important, additional tool in the chemical control of the pathogen.

2. Studies on the base-line sensitivity of *Botryotinia fuckeliana* (de Bary) Whetzel against CGA 173506

2.1 Introduction

Gehmann et al. (1990) was first to report the use of the phenylpyrrole fungicide, CGA 173506; common name n.n. trade mark Saphire®. In contrast to fenpiclonil (CGA 142705) of the same chemical class which is used for seed treatment (Nevill et al., 1988), CGA 173506 has been developed as a foliar fungicide for grapes, stone fruit, vegetables, rice, field crops and turf and as a seed treatment for cereal and non-cereal crops. As a foliar fungicide it provides a high inherent activity against species of the genera: *Botryotinia, Monilinia, Sclerotinia, Rhizoctonia* and *Alternaria*. In the laboratory, Gehmann et al. (1990) did not find cross resistance to methyl-benzimidazole-carbamates, dicarboximides or guanidines when they investigated field resistant strains of *B. fuckeliana*.

Martinetti (1986) and Leroux (1991) reported parallels in the effect of fenpiclonil and the dicarboximide fungicides on *B. fuckeliana in vitro*. Leroux (1991) concluded from his results, that the mode of action of the phenylpyrrole fungicides and of the dicarboximides is the same. However, the mode of action of the phenylpyrrole fungicides has not been clarified yet, and concerning the mode of action of the dicarboximide fungicides, numerous contradictory results have been described in the literature (Corbett et al., 1984, Endlich and Lyr, 1987, Ruiz Borge, 1988).

During the development of novel fungicides, information on the potential of the target organisms to develop resistance under both laboratory and field conditions is required (Georgopoulos and Dekker, 1982, Löcher and Lorenz, 1991). Georgopoulos (1982), Georgopoulos and Dekker (1982), Brent (1988) and Brent et al. (1990) emphasized the importance of determining natural sensitivity of field populations prior to the use of a new fungicide, i.e., the base-line sensitivity. The aim of the following investigation was to determine such base-line sensitivity data of *B. fuckeliana* concerning CGA 173506. In comparison with the results reported by Martinetti (1986) and Leroux (1991), working with fenpiclonil and a dicarboximide fungicide, the effects of CGA 173506 and vinclozolin on the mycelial growth and on the germination process of conidia were compared.

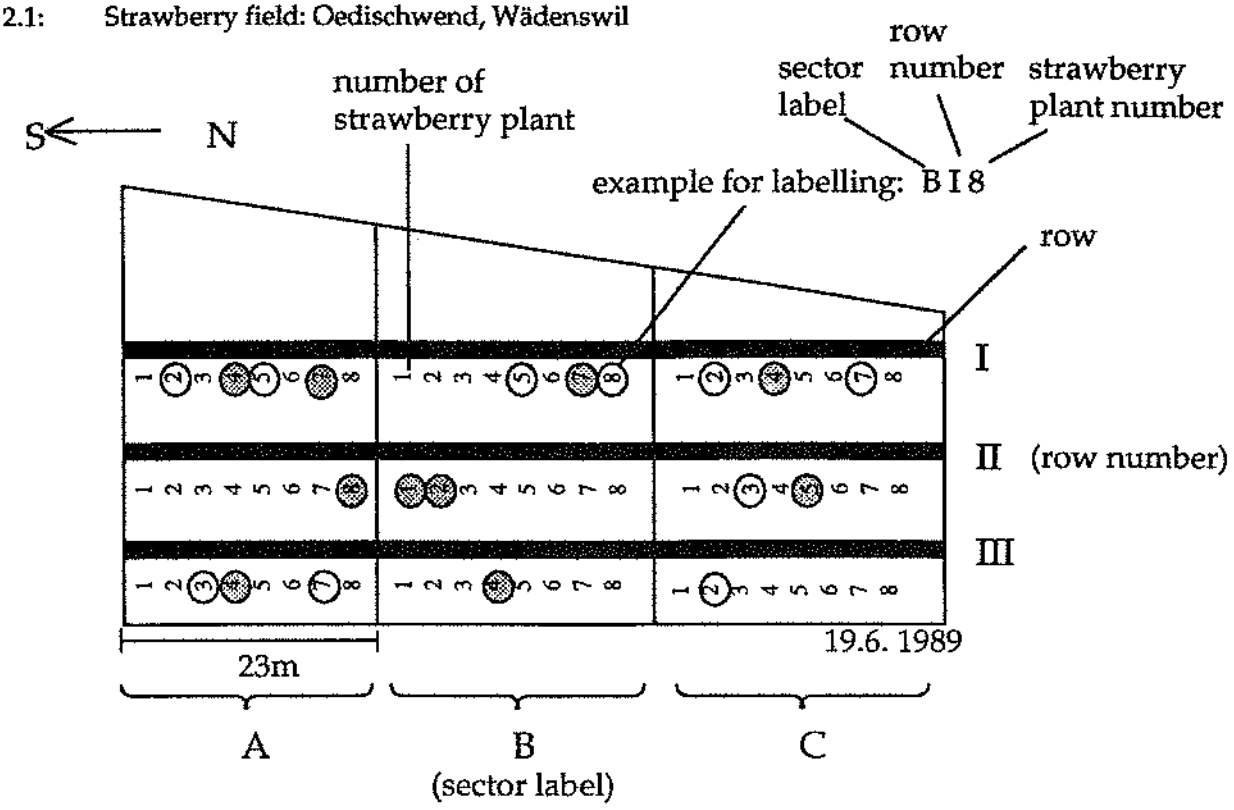
2.2 Materials and Methods

Isolation of *B. fuckeliana*

Isolates from naturally infected strawberries of different varieties including Tenira were collected from fields in Oedischwend, Wädenswil (Fig. 2.1) and in Fondli, Dietikon (Fig. 2.2). In none of the fields had any fungicides been applied. The field in Oedischwend consisted of three rows, with a total length of 70m each. The rows were numbered from I to III. Each row was divided in three sectors A, B and C each consisting of 56 plants. Eight strawberry samples, infected with *B. fuckeliana*, were taken per row and sector. These samples were numbered from 1 to 8. Each isolate could thus later be localized by the row number, the sector number and the sample number of the strawberry from which it was isolated. The field in Fondli consisted of 5 rows (I to V) which were divided in three sectors (X, Y and Z). From 20 plants per row and sector, two infected strawberry samples were taken. The nomenclature of the isolates of the samples from Fondli was equivalent to the nomenclature for isolates from Oedischwend.

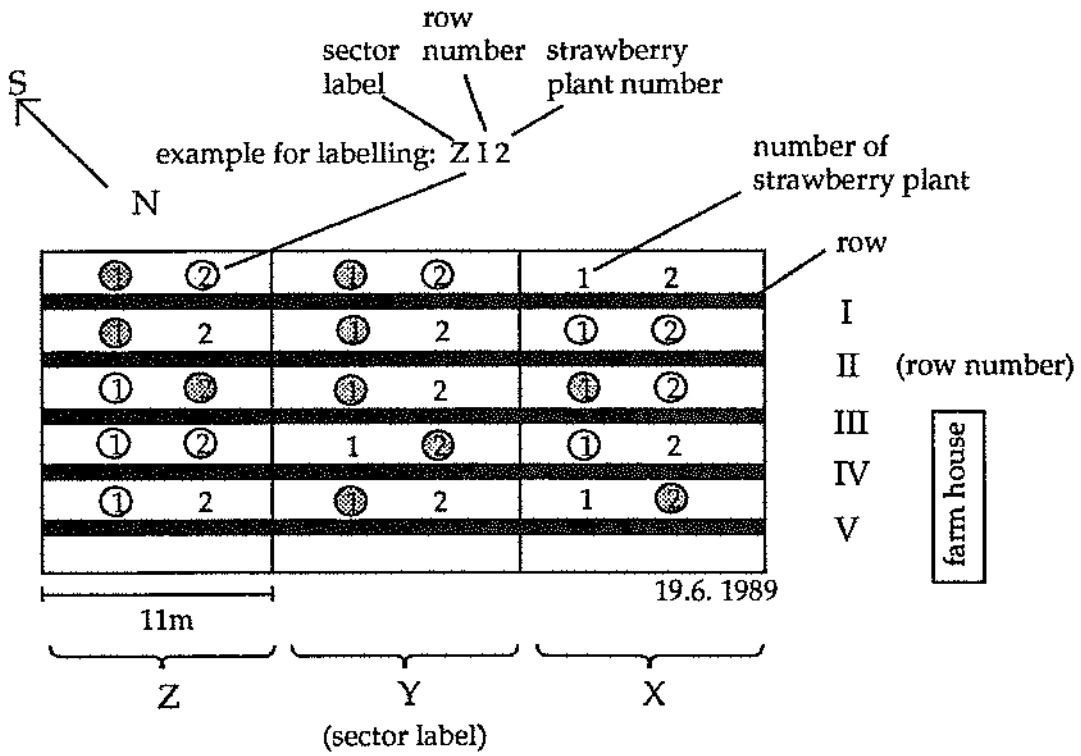
Strawberries which were latently infected with grey mould were incubated with a wet cotton plug at 20°C

Fig. 2.1: Strawberry field: Oedischwend, Wädenswil



20 isolates (○ and ◍) were stored in liquid nitrogen
10 isolates (◍) were selected for further experiments

Fig. 2.2: Strawberry field: Fondli, Dietikon



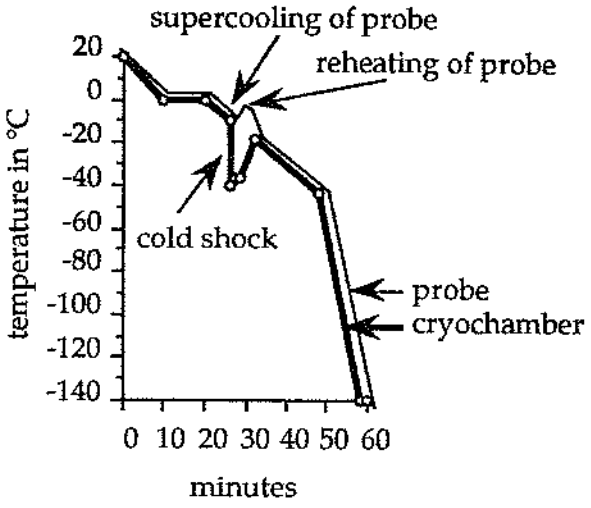
20 isolates (○ and ◍) were stored in liquid nitrogen
10 isolates (◍) were selected for further experiments

for several days to enhance sporulation. Conidia were transferred to malt agar (12g agar-agar, Merck, 15 malt extract, Merck) amended with 100mg streptomycin/L and 100mg oxitetracyclin/L. Colonies growing on agar amended with antibiotics were transferred onto pea agar (160g frozen green peas, Coop, 20g agar-agar, Merck and 5g saccharose, Merck in 1L distilled water, pH = 6) and incubated for 7d at 20°C in 12h dark, 12h fluorescent light (Osram L 20W/25S) and black light (Philips TLD 18W/08).

Cryopreservation of *B. fuckeliana* in liquid nitrogen

The method used for cryopreservation of young mycelium from *B. fuckeliana* is adopted from Dahmen et al. (1983) and from Hohl and Iselin (1987). Spore suspensions of isolates from Oedischwend, Wädenswil were adjusted to 10^5conidia/ml. Malt agar discs of a diameter of 10mm were inoculated with 50µl of the spore suspension and then incubated for 17h at 20°C in the dark. Three to five discs containing young mycelium of 17h were put into 2ml plastic cryotubes (NUNC, Kamstrup, Denmark); skim milk (17% skim milk, 20% glycerol, Difco, Merck in a mixture 1:1) was added as a cryoprotectant to each cryotube up to the mark at 1.8ml. Ten cryotubes for each of 20 strains of *B. fuckeliana* from Oedischwend, Wädenswil (encircled in Fig. 2.1) and from Fondli, Dietikon (encircled in Fig. 2.2) were cryopreservated in liquid nitrogen. For controlled freezing a programmable liquid nitrogen freezing unit (Planer Programmable Temperature Controller PTC 303, Sunburry on Thames, England) was used. Fig. 2.3 shows the development of temperature in the cryochamber and in the probe during the controlled cryopreservation process. Reheating of the supercooled probes during the freezing process were minimized by using the freezing program described for Phytophthora species by Hohl and Iselin (1987). It provided an averaged cooling rate of 1.5°C/min. For thawing, the cryotubes were removed from the storage tank and warmed up at room temperature for 20 to 30 minutes. The agar discs were then aseptically transferred to either malt agar plates or to test tubes containing pea agar.

Fig. 2.3: Diagram of temperature development of the cryochamber and the probe during a controlled freezing process



Fungicides and media

Vinclozolin and CGA 173506, both technical grades, were provided by Ciba-Geigy Ltd.. From stock solutions, prepared by dissolving 300mg and 100mg vinclozolin or CGA 173506, respectively, by ultra sound for 5 minutes at room temperature in 100ml ethanol abs. further stock solutions of 0.3mg to 30mg vinclozolin and of 0.01 to 30mg CGA 173506/100ml ethanol abs. were obtained by 10 fold dilutions. The stock solutions were stored at 0°C and were reprepared at least every third month.

For agar plate assays, media amended with fungicides were prepared adding 10ml of stock solutions of vinclozolin or CGA 173506, respectively, to autoclaved (120°C, 101,2kPa, 20 minutes), and cooled (50°C) malt agar (12g agar-agar and 15g malt extract/L, Merck). For final concentrations of 100mg

vinclozolin/L, 100mg of the active ingredient were dissolved in 10ml ethanol abs. and then added to the medium.

Sensitivity to dicarboximide fungicides

To ensure that all isolates (encircled in Fig. 2.1 and Fig. 2.2) were sensitive wild types, their reaction on dicarboximides was determined roughly by placing 10mm mycelial discs (17h old mycelium) on malt agar amended with 0, 0.3, 1 and 3mg vinclozolin/L. Colony diameters were assessed after an incubation period of 3 days at 20°C in the dark. Colonies on media amended with vinclozolin that grew more than half of the diameter of the controls containing no fungicide were classified as resistant at the appropriate level.

Dose-response relationship of vinclozolin and CGA 173506

Ten vinclozolin sensitive strains, from both locations each, were chosen from the twenty strains which were cryo-preservated in liquid nitrogen (shaded circles in Fig. 2.1 and Fig. 2.2). The dose-response relationship of both ingredients was assessed for the germination process of conidia and for mycelial growth of young mycelium. The base-line sensitivity concerning both ingredients was determined for the populations of Oedischwend and Fondli. The reaction of the young mycelium (17h old) was determined in an agar plate test by placing three discs containing the mycelium upside-down on malt agar (diam. 8cm) amended with 0, 0.03, 0.1, 0.3, 1, 3 and 10mg vinclozolin/L or 0,001, 0.003, 0.01, 0.03, 0.1, 0.3, and 1mg CGA 173506/L, respectively. Mycelial growth was assessed by measuring a mean colony diameter after an incubation period of three days at 20°C in the dark. The diameter of the inoculation disc (10mm) was subtracted from each value to give the true value that was compared to the control plates containing 10ml ethanol abs. but no fungicide. For the germination test 50µl of a suspension of 10^5 conidia/ml were placed on malt agar discs amended with 0, 0.1, 0.3, 1, 3 and 10 mg vinclozolin/L or 0.01, 0.03, 0.1, 0.3 and 1 mg CGA 173506/L, respectively. Germination rates of conidia that have developed germ tubes which were at least as long as the conidia, were assessed after an incubation period of 13h at 20°C in the dark. Percentage of inhibition of growth and germination, respectively, on agar amended with fungicides were determined by reference to the controls. Values of EC50 were determined graphically.

2.3 Results

Sensitivity to dicarboximide fungicides

Of the twenty isolates from Oedischwend, Wädenswil all but two were sensitive to vinclozolin. Their growth rate was less than half of the control on agar amended with 0.3mg vinclozolin/L. Strains AI5 and

Table 2.1: Number of isolates growing less than 50% of the control on agar amended with 0.3, 1 and 3mg vinclozolin/L (*AI5 and BI5, °XIV1, XV2 and ZV1)

	Number of isolates with EC50 values for mycelial growth			
origin of isolates	< 0.3mg vinc./L	0.3-1mg vinc./L	1-3mg vinc./L	> 3mg vinc./L
Oedischwend, Wädenswil	18	0	2*	0
Fondli, Dietikon	17	3°	0	0

Fig. 2.4: Inhibition of conidia germination: Dosage-response relationship of CGA 173506 and of vinclozolin in 10 strains from Oedischwend, Wädenswil and from Fondli, Dietikon, respectively

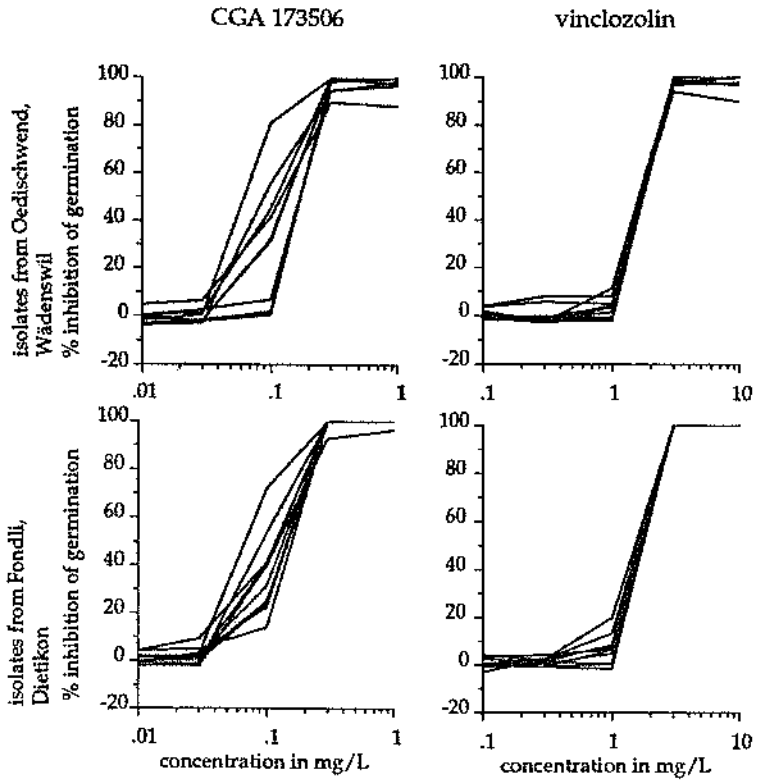


Fig. 2.5: Inhibition of mycelial growth: Dosage-response relationship of CGA 173506 and of vinclozolin in 10 strains from Oedischwend, Wädenswil and from Fondli, Dietikon, respectively

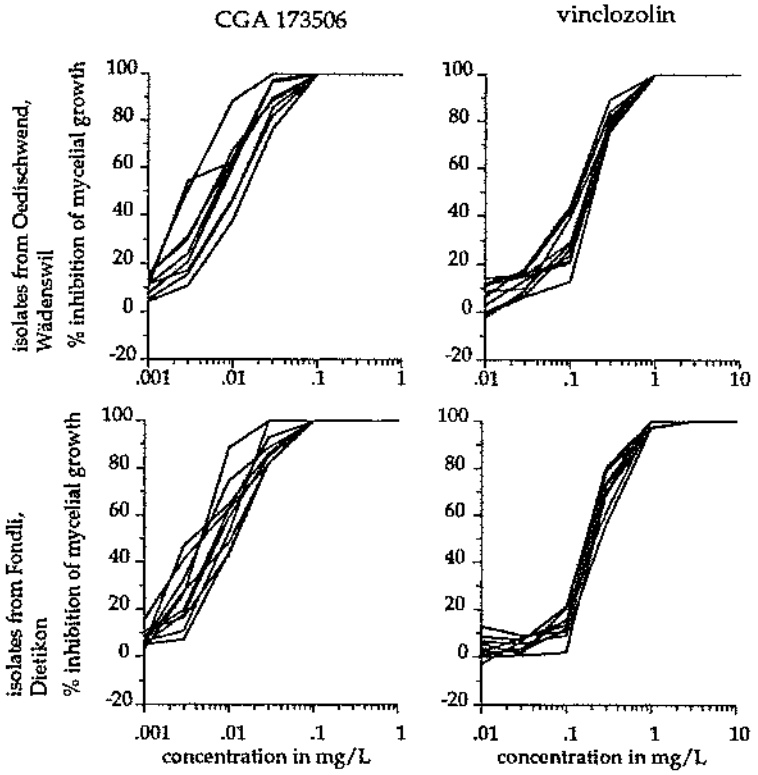


Fig. 2.6: Distribution of EC50 values of ten wild type strains from Oedischwend and from Fondli, respectively; minimum and maximum values are indicated for each combination

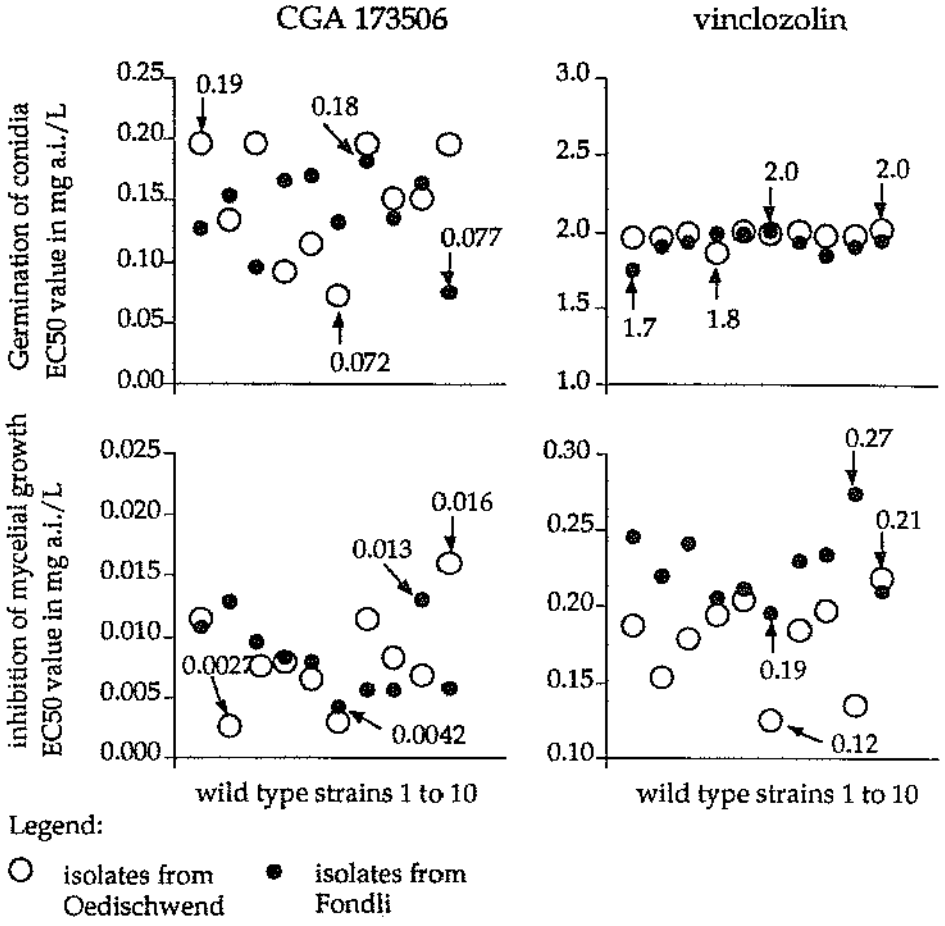


Table 2.2: Response of *B. fuckeliana* to vinclozolin and to CGA 173506, respectively, in a spore germination test and a mycelial growth test

	EC50*			
	spore germination test		mycelial growth test	
	vinclozolin	CGA 173506	vinclozolin	CGA 173506
Oedischwend				
mean (stdev)	1.9 (0.04)	0.15 (0.047)	0.17 (0.03)	0.0092 (0.0037)
range	1.8-2.0	0.072-0.19	0.12-0.21	0.0027-0.016
Fondli				
mean (stdev)	1.9 (0.08)	0.14 (0.033)	0.22 (0.02)	0.0084 (0.0031)
range	1.7-2.0	0.077-0.18	0.19-0.27	0.0042-0.013

*values are means (stdev) in mg/L of 10 strains

BI5, were therefore classified as vinclozolin field resistant, growing more than 50% on 1mg but less than 50% of the control on agar amended with 3mg vinclozolin/L (Table 2.1). All the twenty strains isolated from strawberries from Fondli, Dietikon were sensitive to vinclozolin, their growth rate being less than half of the control on agar amended with 1mg vinclozolin/L. Of the twenty strains, seventeen had a growth rate less than 50% of the control on agar amended with 0.3mg vinclozolin/L whereas strains XIV1, XV2 and ZV1, grew more than half of the control on 0.3 but less than half on agar amended with 1mg vinclozolin/L (Table 2.1).

Dose-response relationships

On agar amended with increasing amounts of CGA 173506 or vinclozolin, respectively, response of *B. fuckeliana* to both fungicides was determined in a mycelial growth test and an all-or-none response was indirectly determined in a spore germination test. As shown in Figures 2.4 and 2.5 dose-response curves are similar in shape for vinclozolin and for CGA 173506. Both ingredients show a strong inhibitory effect on mycelial growth but CGA 173506, however, has shown a higher inherent activity than vinclozolin. For the spore germination test, the minimal inhibitory concentration (MIC) was 3mg/L for vinclozolin and 0.3mg/L for CGA 173506 (Fig. 2.4), respectively, whereas in the mycelial growth test it was 1mg/L for vinclozolin and 0.1mg/L for CGA 173506 (Fig. 2.5).

In the germination test the mean EC50 values of the two populations of Oedischwend, Wädenswil and Fondli, Dietikon, both represented by 10 strains, did not differ significantly (t-test, $p>0.05$). However, in the mycelial growth tests on agar amended with either vinclozolin or CGA 173506 the mean EC50 values of the two populations differed significantly (t-test, $p<0.05$) for vinclozolin but not for CGA 173506 (Table 2.2 and Fig. 2.6). Although the statistically sig-

Table 2.3: List of EC50 values of sensitive wild type isolates of *B. fuckeliana* evaluated by various authors

EC50 values in mg/L		
conidia germination	mycelial growth	author
0.8	0.1-0.3 (0.45)	Albert, 1979
12	0.17	Pappas and Fischer, 1979
1.4	0.1	Pappas, 1982
-	0-1	Lorenzini, 1983
-	0.1-0.2	Katan, 1982, 1985
-	0.15	Leroux and Besselat 1984, 1985
5.3-16	0.11-0.36	Davis and Dennis, 1981
-	0.12	Wang et al., 1986
0.86	0.19	Beever et al., 1989
0.45-0.84	0.04-0.22	Rewal et al., 1991
1.7-2.0	0.12-0.27	Hilber, 1992 (see table 2.2)

nificant difference concerning mycelial growth on agar amended with vinclozolin (Fig. 2.6. and Table 2.2) was not explicable, for further studies the strains from Oedischwend, Wädenswil, which showed the lower mean EC50 value were used.

Base-line sensitivity of CGA 173506 and vinclozolin
Fig. 2.6 shows the distributions of the EC50 values of vinclozolin and CGA 173506 evaluated for 10 strains from Oedischwend, Wädenswil and for 10 strains from Fondli, Dietikon. Minimum and maximum values are indicated by arrows. In the two populations Oedischwend and Fondli, the base-line sensitivity for vinclozolin ranked between 1.7 and 2.0mg/L and for CGA 173506 from 0.072 to 0.19mg/L, respectively, for the germination of sensitive wild type conidia. In the mycelial growth assay the base-line sensitivity ranked between 0.12 and 0.27mg/L for vinclozolin and between 0.0027 and 0.016mg/L for CGA 173506, respectively (Table 2.2).

2.4 Discussion

The strawberries from Oedischwend, Wädenswil and from Fondli, Dietikon were cultivated without application of fungicides and were sold as "pick your own" on the field. Grey mould was indirectly mechanically controlled by the consumers who not only picked healthy fruits for consumption but also had to remove macroscopically visible infected fruits from the plants. Although no dicarboximides at all had been applied in Oedischwend, Wädenswil, from twenty tested samples, two were found to be vinclozolin field resistant. Due to this unexpectedly high frequency of field resistant strains, 40 samples were tested additionally. None of these showed a decreased sensitivity to dicarboximides indicating the resistance frequency to be much lower than it was first anticipated.

Both farmers bought part of their young strawberry plants from Häberli AG, Switzerland where several pesticides were applied to the young plants before they were sold. For the cv. Tenira the treatments included vinclozolin. Strawberries cv. Tenira were only sold to the farmer in Oedischwend, Wädenswil, where the two field resistant isolates were detected, but not to the farmer from Fondli, Dietikon, where no strains were found with a decreased sensitivity to vinclozolin. By applying only a few treatments of dicarboximide fungicides in the field the selection for resistant strains is very rapid as shown in the previous chapter for the vineyard in Walenstadt, Switzerland. The minute, but statistically significant, differences between the mean EC50 values of vinclozolin for mycelial growth in the two populations Oedischwend, Wädenswil and Fondli, Dietikon are of no practical relevance. However, because of the treatments with vinclozolin during the production of young plants, strains of *B. fuckeliana* that were resistant to dicarboximides in the field survived during the season and could be detected. This indicated the presence of isolates with nuclei carrying the gene for resistance to dicarboximides. Although, the proportion of isolates carrying nuclei with the resistance gene is very small in population of Oedischwend, Wädenswil, the influence of the resistance gene cannot be completely excluded.

The EC50 value is a general purpose value (Brent, 1988) which, commonly, is graphically determined by plotting percentage inhibition of mycelial growth against the logarithm of fungicide concentration (Grindle, 1983, Lorenzini, 1983, Beever et al., 1989, Köller and Wubben, 1989). The precision of its determination, however, depends on the number and the range of concentrations that are used to assess the dose-response relationship. Due to practical reasons the number of concentrations used to evaluate dose-response curves is limited. Probit analysis does often not satisfy mathematically as frequently too few values are available for a reliable regression analysis. Even though EC50 values are generally used to

characterize dose-response relationships the aforementioned reservations have to be considered.

To assess the base-line sensitivity of CGA 173506 and of vinclozolin in *B. fuckeliana* EC50 values of 10 strains from each of two populations were graphically determined from dose-response curves. In table 2.3 the results for vinclozolin are compared with data given in the literature. Buchenauer (1976), Albert (1979), Pappas and Fisher (1979), Pappas (1982), Davis and Dennis (1981), Beever (1989) and Rewal et al. (1991) found the spore germination process much less affected by vinclozolin than the mycelial growth. As indicated in Fig. 2.4 and 2.5 in table 2.2 the same effect could be demonstrated for the phenylpyrrole compound CGA 173506. Concerning mycelial growth the range of EC50 values of vinclozolin from sensitive wild type strains indicated in Fig. 2.6 agrees with the data given in the literature (Table 2.3). However, Buchenauer (1976) found slightly higher values whereas Rewal et al. (1991) found strains with slightly lower EC50 values. Regarding the germination test, the range of EC50 values given in the literature for vinclozolin is much broader (Table 2.3). As some conidia produce a non vital germ tube, the evaluation of the conidia germination test is difficult which is reflected in the broad range of EC50 values of vinclozolin determined by different authors.

3. Studies on the occurrence of laboratory resistance against vinclozolin and CGA 173506 in *Botryotinia fuckeliana* (de Bary) Whetzel and its influence on fitness parameters

3.1 Introduction

Most fungi have a short generation time and produce a high number of propagules, among which, assuming a frequency of mutations towards resistance from 10^{-4} to 10^{-10} (Dekker, 1987, Gisi and Csech, 1988a) resistant progenies are likely to occur. Therefore the occurrence of resistant strains in a field population is the rule rather than the exception (Gisi and Csech, 1988a). Brent et al. (1990) assumed the resistance alleles in a natural population to occur below an expected frequency of 10^{-6} due to the often reduced fitness of the resistant pathogens. As fungicide resistance is a major problem in plant disease control (Dekker, 1987), for the development of new products it is essential to study, in an early stage, the potential of the target organisms to develop resistance against the novel active ingredient (Dekker, 1982b, Schwinn, 1982, Gisi and Csech, 1988a,b, Brent et al., 1990, Schwinn and Morton, 1990). To assess the resistance risk it is thus crucial to know the inherent genetic variation in sensitivity towards the fungicide in the pathogen population (Gisi and Csech, 1988b, Brent et al., 1990). Due to genetic flexibility (see p. 6) overall variability can be drastically increased in fungi carrying two or more genetically different nuclei in the same cytoplasm forming a heterokaryon. Anastomosis furthermore allows the free exchange of nuclei within the fungal population. If only few nuclei that carry a gene for fungicide resistance are present in a fungal population the exposure to the appropriate fungicide will conceivably increase the proportion of nuclei which carry the gene encoding for resistance (Georgopoulos, 1987). *B. fuckeliana*, due to its biology, is therefore a pathogen "at risk" carrying a high potential to develop fungicide resistance to new chemical compounds.

Gisi and Csech (1988a,b) and Brent et al. (1990) who refer to the aforementioned authors, recently published resistance risk evaluation schemes for new candidate chemicals to be used in disease control. The overall resistance risk is split into inherent risk and management risks. The inherent risk is comprising parameters concerning the effect of the fungicide on the pathogen with respect to its biology and to the environmental conditions whereas the management risk includes the factors related to the practical use of the fungicide (Staub and Sozzi, 1984, Gisi and Csech, 1988a, b). Although emergence of strains that are resistant to single site inhibitors (e.g., dicarboximides, phenylamides, methyl-benzimidazolecarbamates and SBI's) in the laboratory is common, this does not necessarily imply the buildup of a resistant population in the field (Dekker, 1982a, 1987). Nevertheless the occurrence of laboratory resistant strains indicates the risk of resistance development and thus should not be underestimated (Brent et al. 1990). The question whether the fungicide resistance risk in the field can be estimated from laboratory and greenhouse tests is discussed by Dekker (1982a). For the assessment of the inherent risk of a new product it is essential to compare fitness parameters of sensitive wild type strains with strains showing a reduced sensitivity to the new active ingredient.

Based on their preliminary results from studies on the occurrence of resistance in *B. fuckeliana* to fenpiclonil and vinclozolin or iprodione, respectively, Martinetti (1986) and Leroux (1991) assumed that the inherent resistance risks of dicarboximides and phenylpyrroles are similar in this fungus. In the present studies the occurrence of CGA 173506 laboratory resistant strains of *B. fuckeliana* was studied with

regard to changes in relative fitness of resistant strains. Inherent resistance risk parameters were studied and compared for vinclozolin and CGA 173506.

3.2 Materials and Methods

Strains

The occurrence of laboratory resistance was studied in 10 sensitive wild type strains from Oedischwend, Wädenswil. These strains were characterized qualitatively by assessing growth, sporulation, colour and production of sclerotia on potato dextrose agar (39g potato dextrose agar /L, Merck) as well as quantitatively by measuring daily increase in colony diameter on malt agar and virulence on apple.

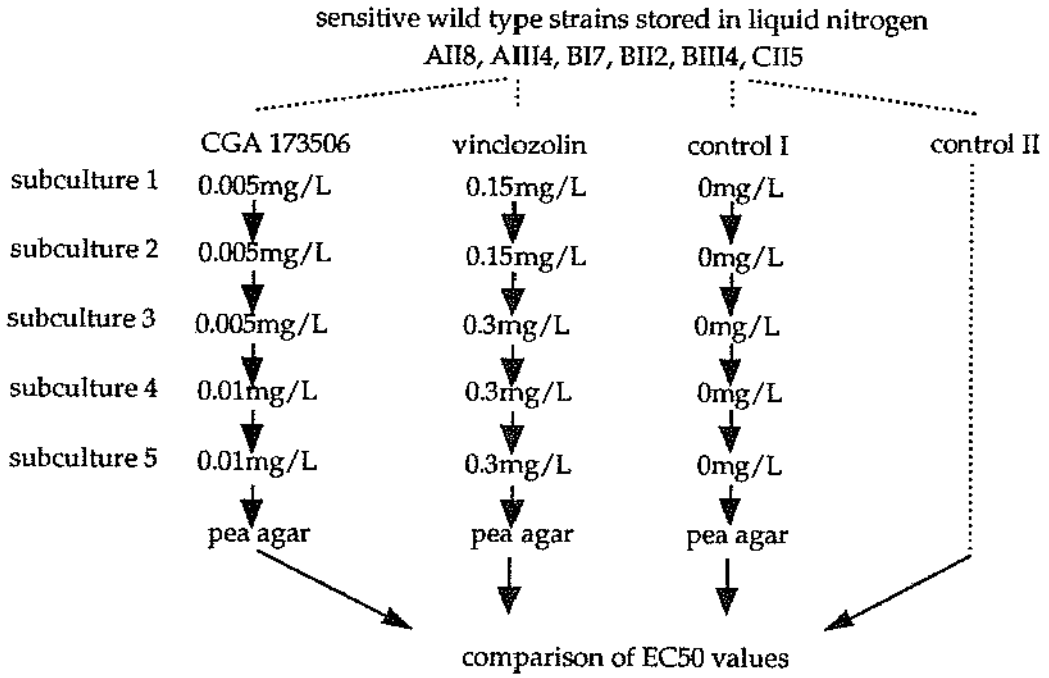
Visual assessment of growth, sporulation, colour and production of sclerotia on potato dextrose agar

Colonies that grew for two weeks at 20°C under fluorescent light in test tubes which contained potato dextrose agar were visually assessed. Growth and sporulation were described as regular and as strong, respectively, when the surface of the potato dextrose agar in the test tubes was completely covered with sporulating mycelium (Table 3.1). Irregular sporulation (agar only partly covered with sporulating mycelium) was described as medium (Table 3.1). The colours of the colonies shaded off in pale grey, grey, dark grey and brown grey. Production of sclerotia was assessed as either yes or no.

Growth rates

Spore suspensions of 10^5conidia/ml were prepared from sporulating cultures on potato dextrose agar. Suspensions (50µl) were added to malt agar discs (diam. 10mm). The discs were incubated at 20°C for 17h in the dark and then either used in a pathogenicity assay (see p. 26) or placed upside-down in the centre of malt agar plates (diam. 15cm) to assess mycelial growth. The mean colony diameter of three replicates were measured during 6 days. Minimal and maximal daily increase in diameter and mean daily increase over 5 days were calculated.

Fig. 3.1: Adaptation on sublethal concentrations of vinclozolin and CGA 173506; scheme of subcultures on agar amended with 0.005 to 0.01mg CGA 173506/L or 0.15 to 0.3mg vinclozolin/L



Pathogenicity assay

The pathogenicity assay was adopted from Schüepp and Küng (1978). Apples cv. Golden Delicious were divided in half, surface sterilized with ethanol 70% and circularly cut-injured with a cork borer at three places on each half. The circularly cut parts were removed and replaced by mycelial discs. As a control treatment, the removed parts of the cuticle were replaced by sterile malt agar discs. Diameters of the rot lesions were assessed after an incubation period of 3 to 4 days at 20°C in the dark. Each wild type strain was tested in 6 replicates.

Adaptation test

In an adaptation test 6 sensitive wild type strains were subcultured on malt agar amended with 0.005 or 0.01mg CGA 173506 and 0.15 or 0.3mg vinclozolin/L, respectively (Fig. 3.1). By choosing these concentrations near the EC50 values of the sensitive strains, their mycelial growth was only slightly inhibited. Spontaneously arising sectors did not occur under these conditions. After the 5th subculture the strains were transferred to fungicide free pea agar. The spores produced on pea agar were used for a mycelial growth test on agar amended with 0.03 to 3mg vinclozolin/L or 0.001 to 0.3mg CGA 173506/L, respectively; the dose-response curves of the strains subcultured on agar amended with either CGA 173506 or vinclozolin and on non amended agar, respectively, were assessed and compared to the curves of the original strains that were not subcultured.

Production of "runaways" with CGA 173506 and vinclozolin

Strains spontaneously developing from sensitive wild type strains on agar amended with lethal or strongly inhibiting concentrations of CGA 173506 or vinclozolin, respectively, were termed "runaways". Most of them showed a reduced sensitivity to CGA 173606 and to vinclozolin, and surprisingly some of them, however, proved to be sensitive.

"Runaways" were produced on agar containing fungicides in the concentrations: 1mg vinclozolin/L, 3mg vinclozolin/L, 0.3mg CGA 173506/L or 1mg CGA 173506/L, respectively. As parental strains the aforementioned 10 wild type strains from Oedischwend, Wädenswil were used. Agar discs were inoculated with 50µl from a suspension of 10^6 conidia/ml. After an incubation period of 17h at 20°C in the dark, three of these mycelial discs were placed upside-down into each of 10 agar plates per concentration and parental wild type strain. The plates were further incubated at 20°C in the dark. The appearance of "runaways" was checked every second day. For further characterization "runaways" were transferred to labeled test tubes containing potato dextrose agar. The labels were consisting of 3 parts: the name of the parental strain from which they were derived (A-C/I-III/1-8), the fungicide concentration on which they were produced (0.3Z3*, 1Z3*, 1V** or 3V**; * Z3 = CGA 173506, **V = vinclozolin) and the plate on which they occurred (1-10).

Characterization of "runaways"

Morphological classification

A total of 107 "runaways" that developed spontaneously from the sensitive wild type strains were morphologically classified. Fig. 3.2 shows the 5 morphological classes in which the "runaways" were categorized.

Fast growing "runaways" of types IA, IB, IC and ID spontaneously arose from the mycelial disc. They either grew as circular colonies (IA and IB) or as sectors (IC and ID). Type IA differed from IB in the form of the edge of the colony. In IB the edge frayed out, whereas it was regular in IA. Type IC contrasted with type ID by developing two or more sectors.

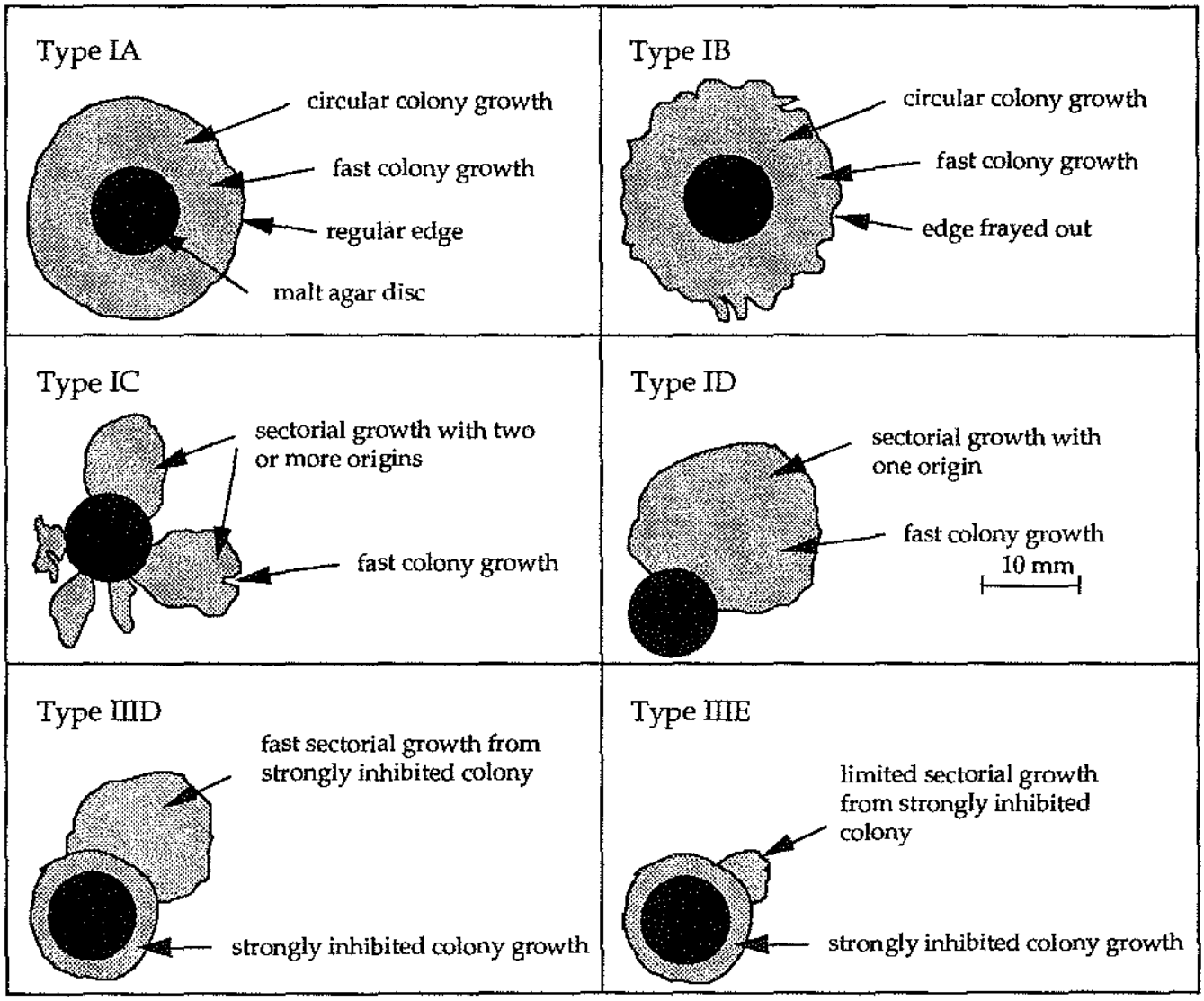
In contrast to the fast growing "runaways" of types IA- ID the "runaways" of types IIID and IIIE

arose from strongly inhibited growing mycelium. Type IIID developed sectors, comparable to the sectors found in ID, whereas IIIE only formed small sectors that were limited in vitality.

Tolerance of vinclozolin and CGA 173506

Mycelial growth on agar amended with 1, 3 and 100mg vinclozolin/L and 0.3, 1 and 3mg CGA 173506/L, respectively, was compared to the mycelial growth on fungicide free medium. Mycelial discs were prepared by either cutting them from the periphery of 4d old cultures or by incubating discs for 17h at 20°C on which 50µl of a suspension of 10^5 conidia/ml were placed. The mycelial discs were placed upside-down on the agar plates. After an incubation period of 3d at 20°C in the dark, the mean colony diameter of three replicates per plate was assessed. The inhibition of mycelial growth compared to the control was calculated. "Runaways" which grew more than 50% of the control were referred to as laboratory resistant at the appropriate level.

Fig. 3.2: Classification of "runaways"; the binary classification consisted of growing types (I and III) and morphologic types (A, B, C and D)



Dose-response relationships and pathogenicity assay

Of a total of 107, 34 "runaways" were chosen to assess dose-response relationships of vinclozolin and CGA 173506 in a mycelial growth test. Malt agar discs (diam. 10mm) were inoculated with 50µl of a suspension of 10^5conidia/ml and incubated for 17h at 20°C in the dark. The discs were placed upside-down on agar amended with 0.1 to 100 mg vinclozolin/L or 0.001 to 10 mg CGA 173506/L, respectively. Inhibition of colony growth compared to the controls on fungicide free agar was calculated. Values of EC50 were determined graphically.

Virulence on apple was assessed in a pathogenicity assay as described for the sensitive wild type strains.

Measuring fungal germ tubes by using an image analyzing system

Virulence on apple and sensitivity to vinclozolin and CGA 173506 were assessed for strains CII5, CII5 1V 10 and CII5 0.3Z3 2 in an apple pathogenicity test and in a mycelial growth test, respectively.

Conidia of the sensitive strain CII5, the low laboratory resistant strain CII5 1V 10 and the highly laboratory resistant strain CII5 0.3Z3 2 were used to develop an accurate and rapid technique to measure the lengths of fungal germ tubes by image analysis. One drop of a suspension, adjusted to $5x10^4$spores/ml, was dispensed onto 10mm diameter agar discs. The discs were incubated for ten hours at 20°C in the dark. Afterwards a drop of methylene blue in lactic acid (0.16%) was added on each disc to stain the germ tubes and to stop further growth. The discs were then stored for 12h at 0°C in the dark. During this time the excess stain solution was absorbed by the agar disc. Seventeen germinated spores were measured per treatment. The experiment was repeated 3 times.

The image analyzing system ASBA® (Wild and Leitz Ltd., Switzerland) detected 256 different shades of gray from 0= black to 255= white (Anonymous, 1988). The intensively blue stained germ tubes contrasted clearly with the agar disc and could be easily detected. The germ tubes were examined by light microscopy (Leitz Diaplan) at 250x magnification. The

Fig. 3.3: A germinated conidium of *B. fuckeliana* on malt agar is stained with methylene blue, the white line within the germ tube represents the skeleton of one pixel (picture element) in width. Fig. 3.3y) is the magnification of the area indicated within dotted lines in Fig. 3.3x).

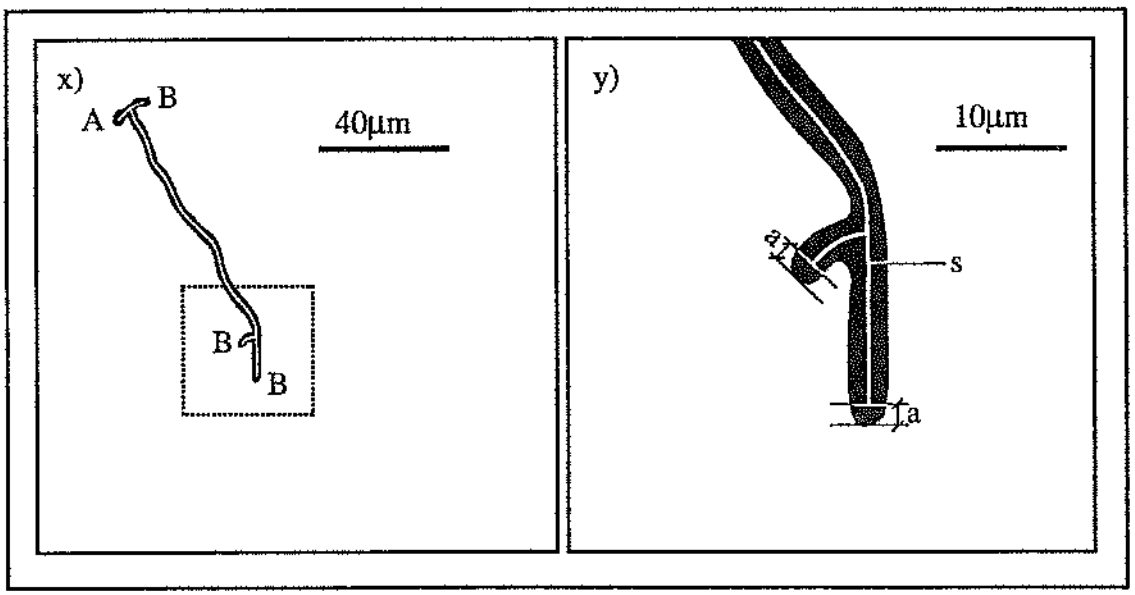


image was recorded by a video camera (Kappa CF6), digitized by the graphic tablet (HIPAD™ digitizer, Model EDT 11 AA P, Houston instruments, Austin Texas) and analyzed by the processor (Z8002).

The software program allowed an image to be reduced in length and width stepwise. For the calculation of the total length, the reducing processes were repeated until the image was reduced to a skeleton of one pixel (picture element) in width (Fig. 3.3). With each step of the reducing process the germ tube image was thus reduced from all sides by two pixels per step. The reduction at the tips therefore amounted to the number of reducing steps times two pixels. The reduction in width was two pixels per step at both sides of the germ tube, which amounted to the number of reducing steps times 4 pixels. When the skeleton of one pixel in width was accomplished, no further reducing of the skeleton occurred. The actual length in µm (l) of the germ tube was calculated by using the following formula: $l = f(na + s)$: where f = translation factor: pixels to µm, n = number of tips (at A and at B shown in Fig. 3.3x), a= the number of reducing steps times two pixels and s= length of skeleton in pixels. The translation factor (f) was the quotient of a measurement in µm of a defined, straight distance measured with the micrometer and of the measurement in pixels of the same distance measured with ASBA®.

Conidial dimensions and number of nuclei per conidium

Conidia of laboratory resistant strains were compared to those of their sensitive wild type parental strains with regard to dimensions. 50µl of a suspension of 10^5 conidia/ml were placed on malt agar discs. Conidia were stained with methylene blue in lactic acid as described for germ tubes previously. Vectorial lengths and widths of a total of 100 conidia were measured interactively by using the image analyzing system ASBA®. For statistical analysis the measurements of all 3 discs were pooled.

Nuclear staining (HCl Giemsa staining and HCl Boroviczeny staining)

The number of nuclei per conidium of sensitive wild type strains was compared with that of the corresponding laboratory resistant conidia.

A modification of the commonly used HCl Giemsa staining method (Menzinger, 1965, Sing, 1983, Akutsu et al., 1983, Lorenz and Eichhorn, 1983, Faretra and Antonacci, 1987, Shirane et al., 1988 and Shirane et al., 1989) was used for the nuclear staining of *B. fuckeliana*. For nuclear staining three drops of a suspension of 10^6 conidia/ml were air dried on a glass slide. For fixation of the conidia the slide was submerged in ethanol glacial acetic acid (3:1) for 10 minutes. The conidia were then washed for 5 minutes each in ethanol 35% and distilled water before they were hydrolyzed for 7 minutes in HCl 10M at room temperature. To remove the excess acid, the slides were quickly washed in distilled water and rinsed in phosphate buffer (P-buffer KH_2PO_4/ Na_2HPO_4; pH= 6.9) for 1 minute. Finally the conidia were stained for 30 minutes in the staining solution (80ml P-buffer, 20ml Giemsa staining solution (Flucka)). The staining procedure was terminated by rinsing the slides in P-buffer to remove excess stain. A cover glass was mounted and the nuclei were counted using a microscope Ortholux II (Wild and Leitz) at magnification 1250x using oil immersion.

In order to verify the stained organelles to be the nuclei, a second DNA binding dye was used. In the HCl Boroviczeny staining method 1g Toluidinblue, 0.5g Safranin, 1g K_2HPO_4, 0.5g KH_2PO_4 and 200ml H_20 were mixed and filled with methanol up to 1L (Reiss, 1965). The staining procedure was the same for the HCl Giemsa staining as for the HCl Boroviczeny staining. The specimens were stained for 20 seconds to 20 minutes in the undiluted stain solution. By the HCl Boroviczeny staining the same organelles as by the HCl Giemsa staining were stained; from this result it was concluded the organelles were nuclei.

Competition test

The competitive ability of 8 laboratory resistant strains of *B. fuckeliana* in mixture with conidia from the corresponding sensitive wild type strains was investigated by assessing the proportion of resistant conidia in the generations following mixed inoculation. A mixture of a dicarboximide field resistant (CGA 173506 sensitive) strain (G 6.15 1.6) and a sensitive (CGA 173506 and vinclozolin) strain (G 6.15 1.8), both derived from single ascospores of the same ascus were additionally included in the experiment. Equal portions of suspensions of 10^3 laboratory resistant and sensitive conidia/ml each were mixed. From every combination 100µl of the mixture were plated on each of 8 malt agar plates containing 0.3mg CGA 173506/L, 3 or 10mg vinclozolin/L, respectively, while 50µl were plated on each of 8 fungicide free plates. Two test tubes containing pea agar were inoculated with 50µl of the same mixture. Colonies growing on fungicide free agar were counted after 2 days whereas colonies growing on agar amended with fungicide were counted after 3 days. Percentage of resistant conidia was calculated (r=100(m/2n); r= % resistant conidia, n= number of counts on fungicide free medium, m= number of counts on medium amended with fungicide). From sporulating cultures on pea agar of the second generation suspensions of 10^3 conidia/ml were prepared. Proportions of resistant conidia were evaluated as described above and 2 fresh test tubes containing pea agar were inoculated. The experiment was run for four generations. Agar plates were incubated at 20°C in the dark; to induce sporulation the test tubes were incubated under white light and black light in a 12h rhythm.

The ability of sensitive, laboratory resistant and field resistant strains to compete on fungicide free pea agar medium was compared with their growth rates and their virulence on apple. Average daily increase in colony diameter was calculated over 3 days. Virulence on apple was assessed as previously described.

Stability of resistance

Stability of laboratory resistance was assessed after successive transfers either from apple over pea agar to apple, or from pea agar to pea agar only. Mycelium of 40 laboratory resistant and sensitive strains was subcultured from pea agar 10 times in irregular intervals during 6 months. In the test including apples 24 laboratory resistant and sensitive strains were transferred from apples to apples via pea agar three times. Dose-response relationships were assessed as described previously. EC50 values of vinclozolin and of CGA 173506 that were determined before and after the transfers over pea agar and over apple, respectively, were compared.

Fitness of laboratory resistant strains as compared to their vinclozolin field resistant parental strains

Five vinclozolin field resistant strains of *B. fuckeliana* were isolated from freshly sporulating grapes in a vineyard in Walenstadt before harvest 1991 (chapter 1). For each strain dose-response curves were determined for vinclozolin and for CGA 173506 in a mycelial growth test. Simultaneously response to increased osmotic pressure and pathogenicity on apple, cv. Golden Delicious, were assessed. To test osmotolerance, mycelial discs containing 17h old mycelium were placed upside-down on malt agar plates amended with 1, 3, 5, 7, 10, 15 and 30% glucose/L. Mean diameters of three replicates per plate were assessed. Mycelial growth on medium supplemented with glucose was compared to the growth of the control on non supplemented medium. "Runaways" of the vinclozolin field resistant strains were produced on agar amended with 10mg CGA 173506/L. The "runaways" were transferred to pea agar; dose- response curves, osmotolerance and pathogenicity on apple were assessed as described for the vinclozolin field resistant parental strains.

3.3 Results

Visual assessment of growth, sporulation, colour and production of sclerotia on potato dextrose agar

As shown in table 3.1 all sensitive wild type strains grew regularly on potato dextrose agar medium and showed medium to strong sporulation. Slight phenotypic variations were visible in the colour of the colonies which were pale grey, grey, dark grey or brown-grey, respectively. Four sensitive wild type strains produced sclerotia on potato dextrose agar whereas no sclerotia formation was observed in the other six strains.

Growth rates and pathogenicity assay

The mean daily increase in colony diameter on an average over 5 days did not differ significantly among 10 strains from Oedischwend, Wädenswil (Kruskal Wallis test p>0,05). As shown in table 3.2 the values of mean daily increase, on an average over 5 days, ranked from 16 to 20mm/day, whereas daily increases ranked from 10 to 32mm/day.

All strains were pathogenic on apple. They caused rot lesions which showed mean diameters that ranked among 17 and 30mm after three days. Controls which were inoculated with sterile malt agar discs did not rot. The virulence differed significantly within the populations as shown in Fig. 3.4.

Adaptation test

In a training experiment the occurrence of a shift in sensitivity to vinclozolin or CGA 173506, respectively, during successive subculturing on media amended with sublethal doses of both fungicides was investigated. Although the mycelial growth of strains BII2 and AII8 was strongly inhibited by 0.3mg vinclozolin/L the formation of "runaways" was not observed. All strains subcultured on agar amended with either vinclozolin or CGA 173506 maintained their sensitivity towards both fungicides. As shown in Fig. 3.5 in this experiment the phenotypic variations with respect to the sensitivity towards vin-

Table 3.1: Visual assessment of characteristics of sensitive wild type strains growing on potato dextrose agar medium

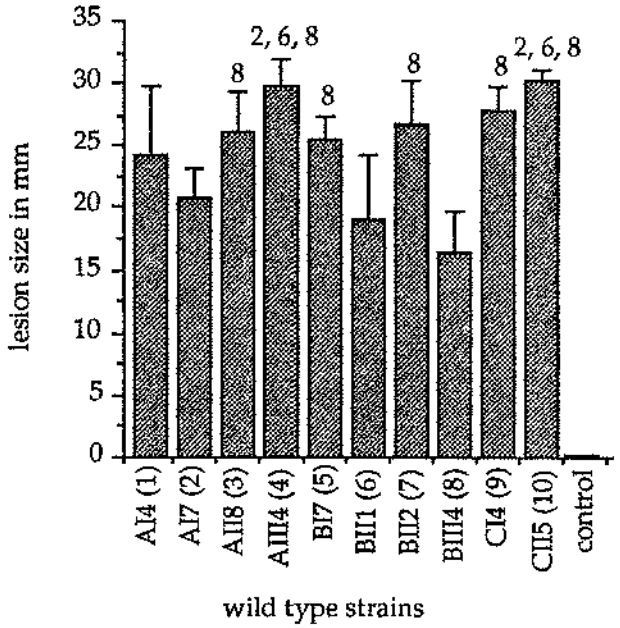
	morphological characteristics on PDA medium			
strains	growth	sporulation	color	production of sclerotia
A I 4	regular	strong	grey-brown	yes
A I 7	regular	strong	pale grey	yes
A II 8	regular	medium	dark grey	yes
A III 4	regular	strong	pale grey	no
B I 7	regular	strong	grey	no
B II 1	regular	medium	pale grey	no
B II 2	regular	medium	dark grey	yes
B III 4	regular	strong	pale grey	no
C I 4	regular	strong	pale grey	no
C II 5	regular	medium	dark grey	no

Table 3.2: Mean daily increase assessed on an average over 5 days and minimal and maximal daily increase in colony diameter

	daily increase in colony diameter in mm			
strains	average over 5 days [a]	stdev	min.	max.
AI4	18	5	11	24
AI7	16	3	10	20
AII8	20	5.6	15	32
AIII4	19	2.3	15	23
BI7	19	5	13	25
BII1	16	3.4	11	21
BII2	19	4.1	12	24
BIII4	16	3.5	12	21
CI4	17	3	12	20
CII5	19	3	15	23

[a] increase in colony diameter was measured in three replicates from the 2nd to the 6th day

Fig. 3.4: Mean virulence of 10 sensitive wild type strains from Oedischwend, Wädenswil, assessed in a pathogenicity assay on apples cv. Golden Delicious. Numbers on top of the bars indicate to which strains significant differences occurred (Anova, $p < 0.05$).



Adaptation test

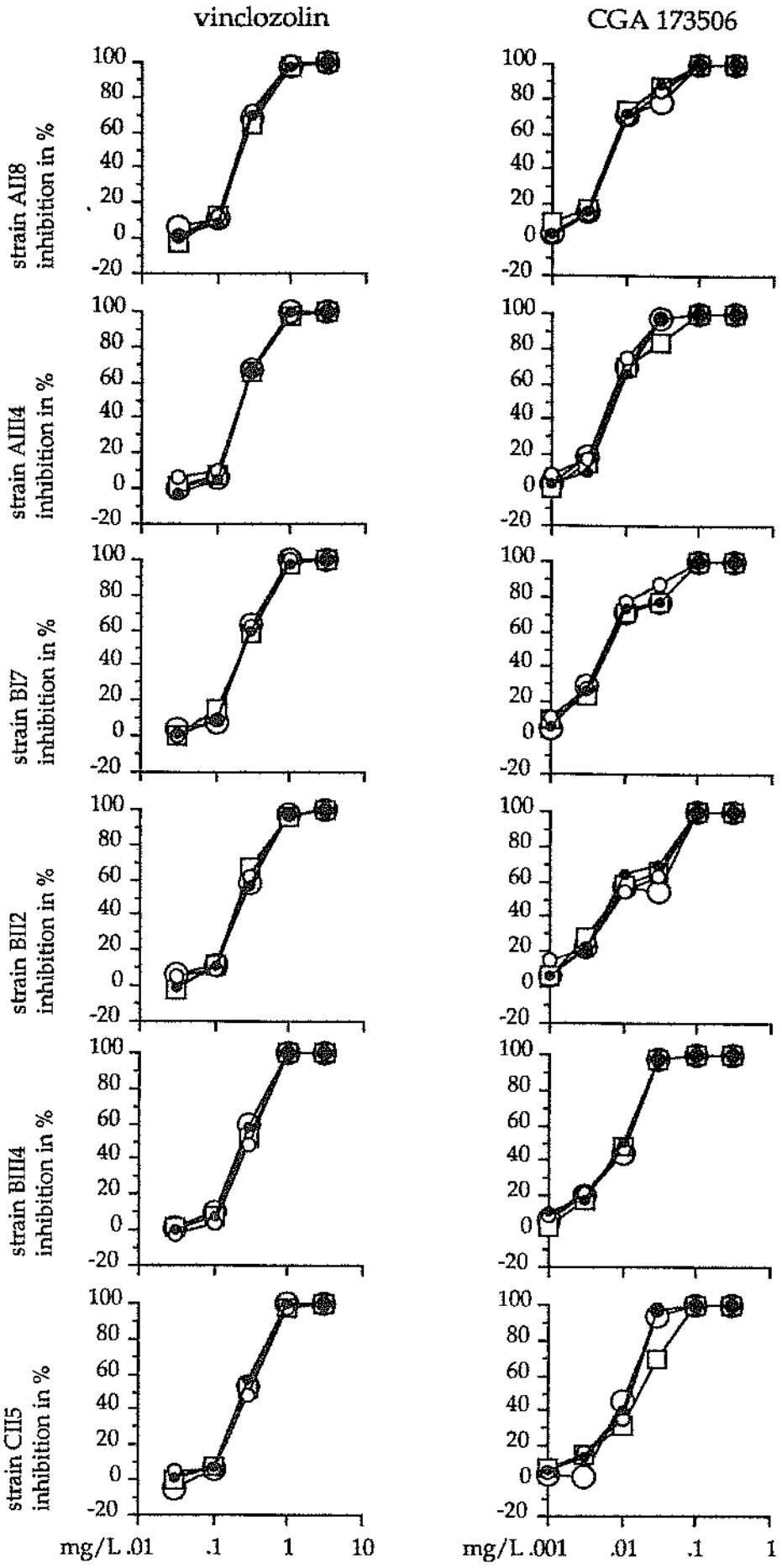


Fig. 3.5:
Adaptation test: comparison of dose-response curves of 6 sensitive wild type strains being stored without subculturing or being subcultured for 5 times on agar amended with 0.005 to 0.01mg CGA 173506/L, 0.15 to 0.3mg vinclozolin/L or on non amended agar, respectively.

Legend:

○ subcultures on fungicide free media

□ no subcultures

○ subcultures on agar amended with 0.15 and 0.3mg vinclozolin/L

● subcultures on agar amended with 0.005 and 0.01mg CGA 173506/L

clozolin and CGA 173506 were minute. Dose-response curves from the cultures which were subcultured for 5 times on fungicide amended media did neither differ from the controls that were subcultured on fungicide free media nor from the dose-response curves from the original strains that were not subcultured.

Production of "runaways" with CGA 173506 and vinclozolin

As shown in table 3.3, 233 "runaways" were produced within 14d. The number of "runaways" produced on agar amended with 0.3 mg CGA 173506/L ranked from 1 to 17, whereas it ranked from 0 to 7 on agar amended with 1mg CGA 173506/L. On agar amended with 1mg vinclozolin/L 1 to 28 and on agar amended with 3mg vinclozolin/L 2 to 20 "runaways" occurred.

Including all types of "runaways" into account, significantly less "runaways" developed on agar amended with CGA 173506 (87), than on agar amended with vinclozolin (146) (contingency table analysis p=0.0001). However, no significant difference (contingency table analysis p=0.75) in the number of "runaways" that arose on either vinclozolin or CGA 173506 amended agar was found when the "runaways" of type IIIE (55) which showed abnormal, restricted growth were excluded.

Characterization of "runaways"

Morphological classification and tolerance of vinclozolin and CGA 173506

Fig. 3.6 describes the distribution of the "runaways" with respect to their morphological type and their sensitivity to vinclozolin and to CGA 173506. The 107 "runaways" could all be ascribed to three patterns:

Pattern 1: EC50 vinclozolin < 1mg/L, EC50 CGA 173506 < 0.3mg/L

Pattern 2: EC50 vinclozolin > 1mg/L, EC50 CGA 173506 > 0.3mg/L and

Pattern 3: EC50 vinclozolin > 1mg/L, EC50 CGA 173506 < 0.3mg/L.

Strains showing EC50 values for CGA 173506 > 0.3mg/L and < 1mg/L for vinclozolin could not be found (Fig. 3.6).

Table 3.3: Number of "runaways" occurring within 14d on agar amended with lethal or strongly inhibiting concentrations of CGA 173506 or vinclozolin, respectively

	number of "runaways" [a]			
parental	CGA 173506		vinclozolin	
strains	0.3 mg/L	1 mg/L	1 mg/L	3 mg/L
AI4	1	3	1	2
AI7	8	7	2	16
AII8	1	4	1	18 (9)
AIII4	3	1	1	4
BI7	4	4	28 (27)	6
BII1	5	0	6 (3)	8 (3)
BII2	3	1	2	4
BIII4	11	2	2	10 (6)
CI4	5	3	2 (2)	20 (1)
CII5	17	4	3	10 (4)

[a] including "runaways" of type IIIE
numbers in () indicate "runaways" of Type IIIE

The EC50 values of the majority of the "runaways" were either > 100mg/L for vinclozolin and > 10mg/L for CGA 173506 (upper end of pattern 2) or < 1 for vinclozolin and < 0.3 for CGA 173506 (pattern 1). Of 40 "runaways", morphologically characterized by the types IA or IB, respectively, 37 were ascribed to pattern 2. From 17 "runaways" of type IIIE, all but 1 were assigned to pattern 1. Nine "runaways" of type IC were ascribed to pattern 2 whereas 3 strains were attributed to pattern 1. "Runaways" of type ID and IIID behaved intermediate.

Dose-response relationships

Dose- response relationships of vinclozolin and CGA 173506 were assessed for 7 strains of pattern 1, 19 strains of pattern 2 and 8 strains of pattern 3. Dose-response curves for both fungicides are given in Fig. 3.7. Concerning vinclozolin two groups of curves were differentiated. The first group included sensitive (VS) and low laboratory resistant strains (VLR), whereas the second group comprised highly laboratory resistant strains (VHR) which showed EC50 values > 30mg/L (Fig. 3.7). The border between sensitive and low resistant strains was arbitrarily set at the EC50 value of 1mg/L. The dose-response curves of CGA 173506 apparently reflected a continuous spectrum, however, the results of genetic analysis in chapter 4 will show that a classification in different sensitivity classes can be made. The resistance level for vinclozolin and for CGA 173506 was identical for all but 3 strains (Table 3.4) which indicates that vinclozolin/CGA 173506 resistances might be coupled. In further experiments, these 3 strains, however, were not stable (data not shown).

Low laboratory resistant strains and field resistant strains apparently showed the same resistance pattern for vinclozolin. Table 3.4, however, shows that in contrast to the vinclozolin field resistant strains in the low laboratory resistant strains decreased sensitivity to vinclozolin was linked to decreased sensitivity to CGA 173506.

Fig. 3.6: Distribution of "runaways" with respect to morphological type and sensitivity to vinclozolin and CGA 173506

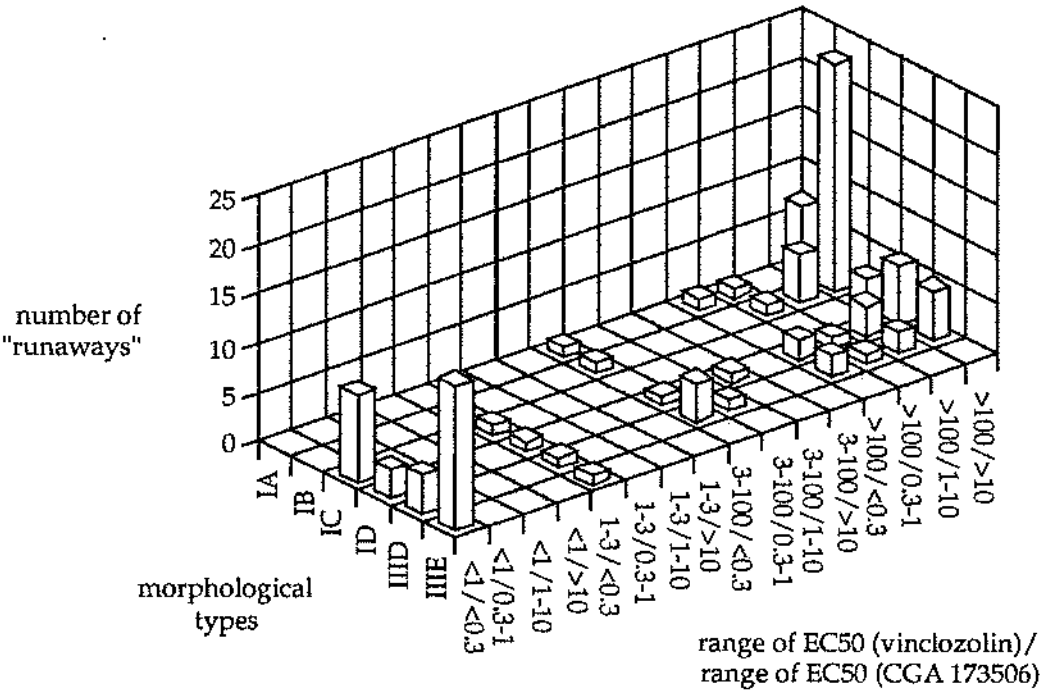


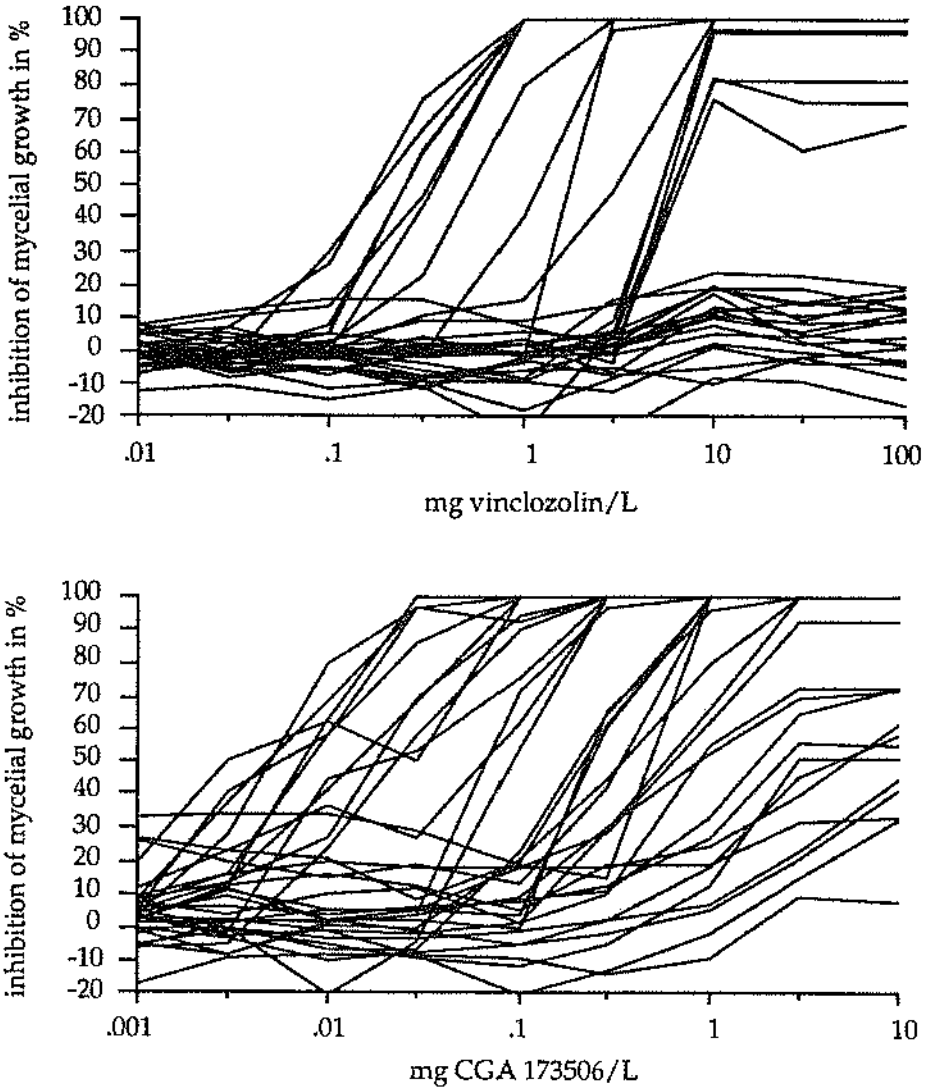
Table 3.4: EC50 values of vinclozolin and CGA 173506 of strains shown in Fig. 3.7

	adopted resistance patterns		EC50 values in mg/L (mycelial growth)	
"runaways"	CGA 173506	vinclozolin	CGA 173506	vinclozolin
BI7 1V 1	CS	VS	0.0057	0.26
BI7 3V 3	CS	VS	0.00855	0.26
BII1 1V 3	CS	VS	0.00655	0.19
BIII4 1V 2	CS	VS	0.00805	0.37
BIII4 3V 7	CS	VS	0.0206	0.62
BIII4 0.3Z3 3	CS	VS	0.00595	0.21
CII5 3V 1	CS	VS	0.0156	0.335
AI4 1Z3 6	CHR	VHR	0.685	> 100
AII8 1V 7	CHR	VHR	0.385	> 100
AII8 3V 1	CHR	VHR	2.05	> 100
AII8 1Z3 4	CHR	VHR	2.9	> 100
AIII4 1Z3 9	CHR	VHR	0.92	> 100
BI7 1Z3 10	CHR	VHR	0.5	> 100
BII1 0.3Z3 6	CLR	VHR	0.25*	> 100
BII2 1V 6	CHR	VHR	6.3	> 100
BII2 0.3Z3 1	CHR	VHR	2.56	> 100
BII2 1Z3 7	CHR	VHR	5.3	> 100
BIII4 3V 6	CHR	VHR	0.75	> 100
CII5 3V 7	CHR	VHR	0.38	> 100
CII5 1Z3 1	CLR	VLR	0.26	7.6
CII5 1Z3 8	CHR	VHR	0.92	> 100
A II 8 0.3Z3 7	CHR	VHR	> 10	> 100
A III 4 3V 2	CHR	VHR	> 10	> 100
AIII4 0.3Z3 1(4)	CHR	VHR	> 10	> 100
BI7 1Z3 1	CHR	VHR	> 10	> 100
CI4 1Z3 10	CHR	VHR	> 10	> 100
AI7 1V 8	CS	VLR	0.0225	3.15**
AII8 3V 6	CLR	VLR	0.03	2
BI7 1V 2	CS	VLR	0.0245	1.35**
BIII4 1V 9	CLR	VLR	0.0945	6.3
BIII4 3V10	CLR	VLR	0.078	6.3
BIII4 0.3Z3 5	CLR	VLR	0.075	6.75
CII5 3V 6	CLR	VLR	0.23	7.25
CII5 0.3Z3 5	CLR	VLR	0.245	7.15

* EC50 value of CGA 173506 was most likely estimated too low
** EC50 value of CGA 173506 was most likely estimated too high

CS CGA 173506 sensitive strains (EC50 < 0.03mg/L)
CLR CGA 173506 low resistant strains (0.03mg/L < EC50 < 0.3mg/L)
CHR CGA 173506 highly resistant strains (EC50 > 0.3mg/L)
VS vinclozolin sensitive strains (EC50 < 1mg/L)
VLR vinclozolin low resistant strains (1mg/L < EC50 < 30mg/L)
VHR vinclozolin highly resistant strains (EC50 > 30mg/L)

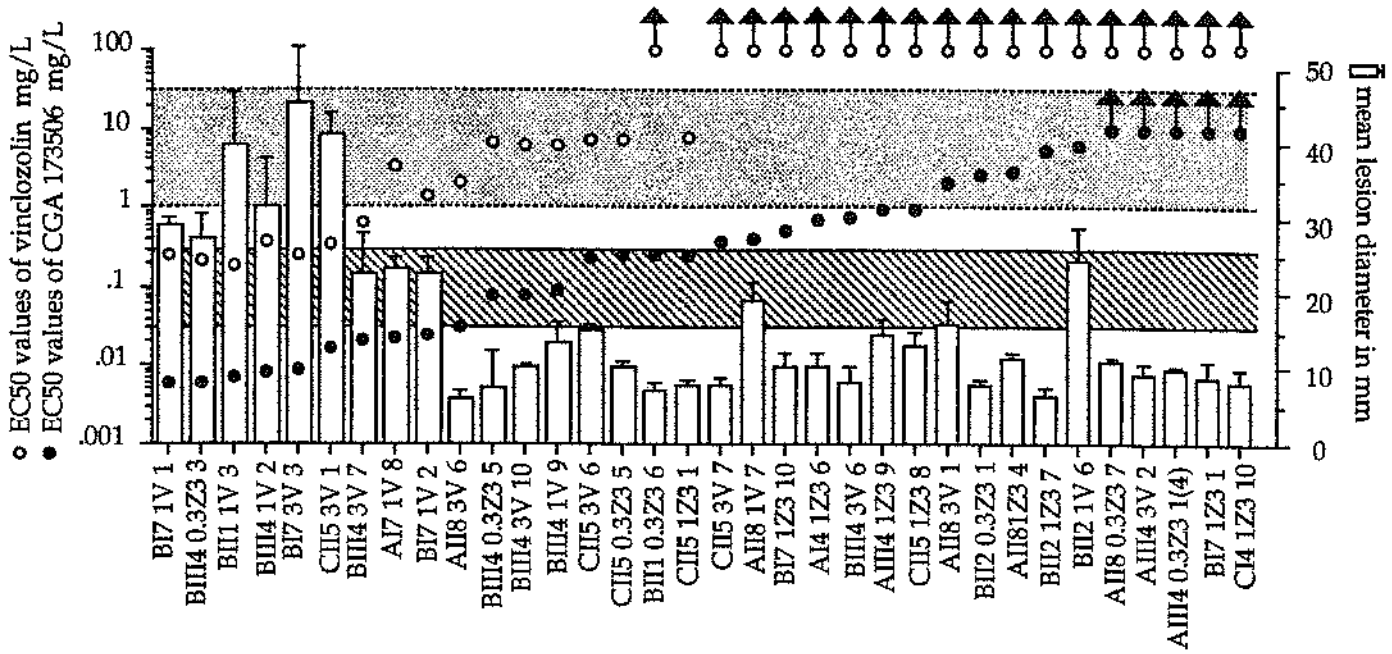
Fig. 3.7: Dose-response curves of vinclozolin and CGA 173506 of 34 selected "runaways"



Pathogenicity assay

The mean virulence on apple and the EC50 values of vinclozolin and CGA 173506 of 34 laboratory resistant and sensitive strains are shown in Fig. 3.8. EC50 values of sensitive, low and highly laboratory resistant strains range within the indicated threshold borders. After an incubation period of 5 days at 20°C sensitive strains caused lesions with diameters that ranged between 22 and 45mm. With the exception of strains AI7 1V 8, BI7 1V 2 and BII2 1V 6 the laboratory resistant strains generally showed a decreased virulence. They caused lesions with diameters less than 20mm. As remarked in table 3.4 strains AI7 1V 8 and BI7 1V 2 are presumably sensitive strains because EC50 values of vinclozolin were found to be < 1mg/L in further tests (data not shown).

Fig. 3.8: Pathogenicity assay of 34 selected "runaways" on apple cv. Golden Delicious



strains arranged according to increasing EC50 values

Legend:

= EC50 vinclozolin > 100mg/L

= EC50 CGA 173506 > 10mg/L

range : vinclozolin low resistant strains

range : CGA 173506 low resistant strains

o EC50 values of vinclozolin mg/L

• EC50 values of CGA 173506 mg/L

stdev of mean

mean lesion diameter in mm (3 replicates)

Measuring fungal germ tubes by using an image analyzing system

Germ tube measurement is often used to study the effect of fungicides on the early development of fungi. Typically a micrometer has been used to measure vectorial lengths of the germ tubes. The micrometer method is feasible only for measuring straight germ tubes. A new technique using the image analyzing system ASBA® was developed for accurate and rapid measurement of fungal germ tubes, independent of their shape. Since an image analyzing system allows inclusion of the curvature in the measurement, minute differences in germ tube lengths can be detected.

In the mycelial growth test strain CII5 was sensitive to both active ingredients, whereas strain CII5 1V 10 was low laboratory resistant and strain CII5 0.3Z3 2 was highly laboratory resistant to both fungicides (Fig. 3.9). In the pathogenicity assay the laboratory resistant strains were less virulent than their sensitive parental strain (Fig. 3.9).

Germ tube lengths of seventeen germinated spores were measured per treatment. The experiment was repeated 3 times. Fig. 3.10 shows the means of the three experiments and their standard deviations.

Fig. 3.9: Dose-response relationship of vinclozolin and CGA 173506 assessed in a mycelial growth test (A and B) and virulence on apple cv. Golden Delicious (C) of strains CII5, CII5 1V 10 and CII5 0.3Z3 2.

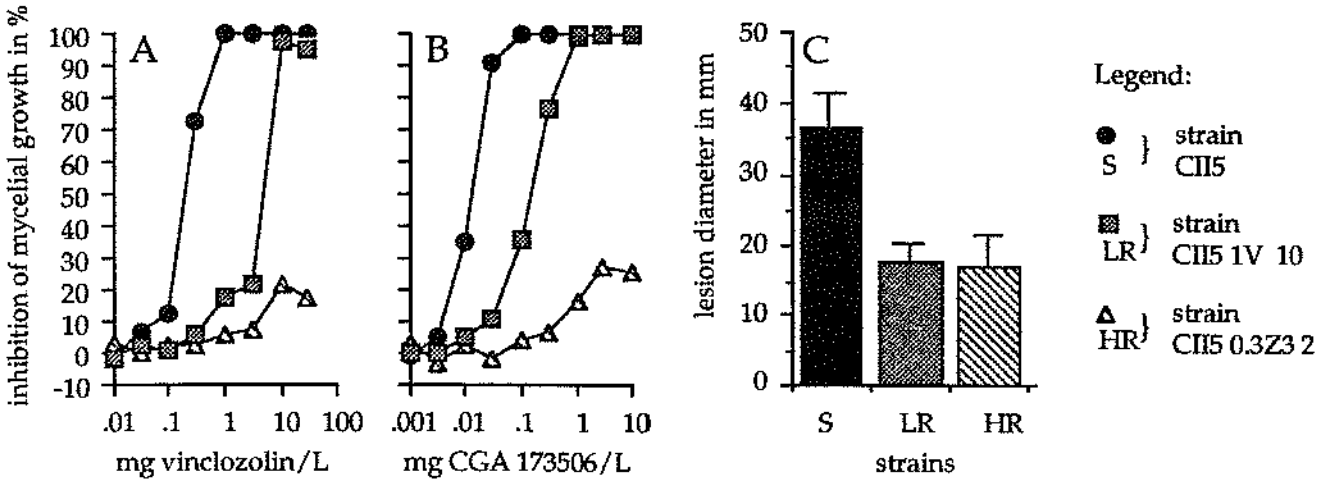
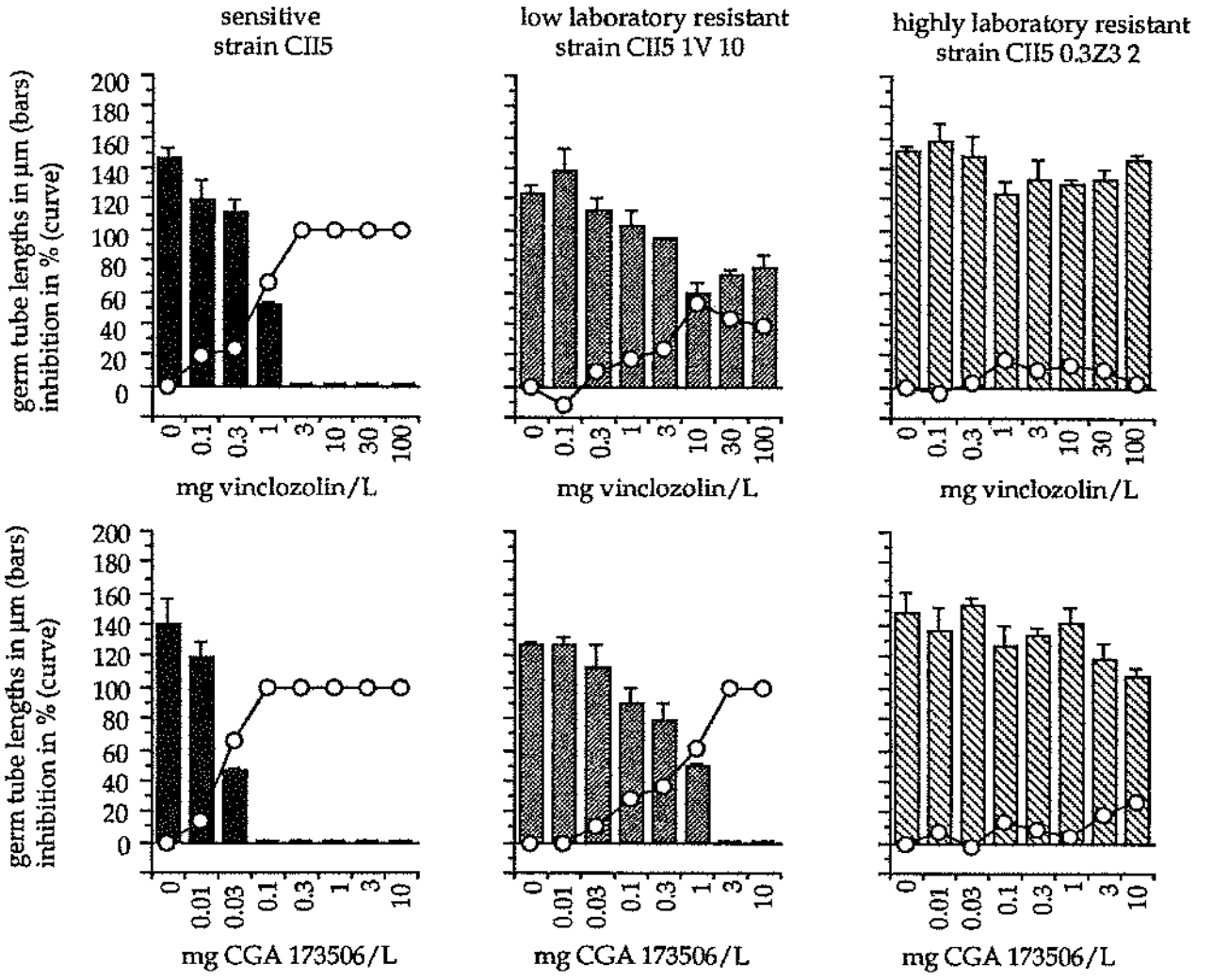


Fig. 3.10: Germ tube lengths (bars) and inhibition of germ tube lengths (curves) of a sensitive, a low and a highly laboratory resistant strain on agar amended with increasing amounts of vinclozolin or CGA 173506, respectively



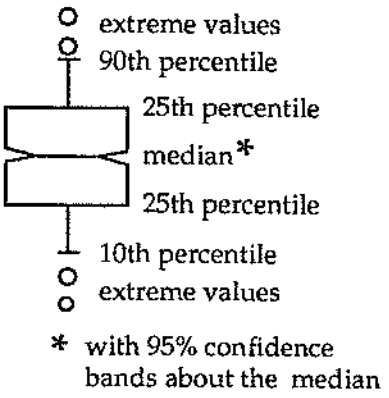
Bars represent the germ tube lengths in µm while curves show the inhibition of germ tube lengths in percentage. Vinclozolin inhibited germ tube growth of the sensitive strain CII5 completely at concentrations of 3mg/L. Germ tube growth of the medium resistant strain, CII5 1V 10, was significantly reduced (Fisher's PLSD test, $p<0.05$) at concentrations of 10mg/L but not at concentrations of 0.1 or 0.3mg/L. There was no statistically significant inhibition of germ tube growth of the highly resistant strain, CII5 0.3Z3 2, at concentrations up to 100mg/L vinclozolin. Germ tube growth of the sensitive strain CII5 was completely inhibited by 0.1mg CGA 173506/L; on agar amended with 0.03mg CGA 173506/L the inhibition was 66%. Strain CII5 1V 10 was significantly (Fisher's PLSD test, $p<0.05$) inhibited at concentrations above 0.01 mg CGA 173506/L. It was completely inhibited by 3mg CGA 173506/L. The germ tube growth of the highly resistant strain CII5 0.3Z3 2 was significantly reduced at the concentrations 0.1, 3 and 10mg CGA 173506/L, at concentrations below 0.1mg/L as well as at 0.3 and 1mg/L the reduction did, however, not differ significantly from the control.

Conidial dimensions and number of nuclei per conidium

Fig. 3.12 shows the frequency distributions of lengths and widths of conidia and the number of nuclei per conidium. Results are shown in notched box plots which display five percentile points (Fig. 3.11).

As shown in Fig. 3.12 no clear trends for differences between laboratory resistant strains and their sensitive parental strains were found. Of 5 resistant strains, derived from the sensitive wild type strain AII8, 3 strains differed significantly in lengths, whereas all 5 differed significantly in widths. There was, however, no difference in the number of nuclei per conidium. Concerning strain AIII4, 2 of 3 laboratory resistant strains differed significantly in lengths and one differed in width. One strain differed significantly in the number of nuclei per conidium. Two laboratory resistant strains, both derived from the sensitive wild type strain BI7 differed significantly in lengths, widths and in the number of nuclei per cell (Mann Whitney U, $p< 0.05$).

Fig. 3. 11: Notched box plots, description of 5 percentile points of a frequency distribution

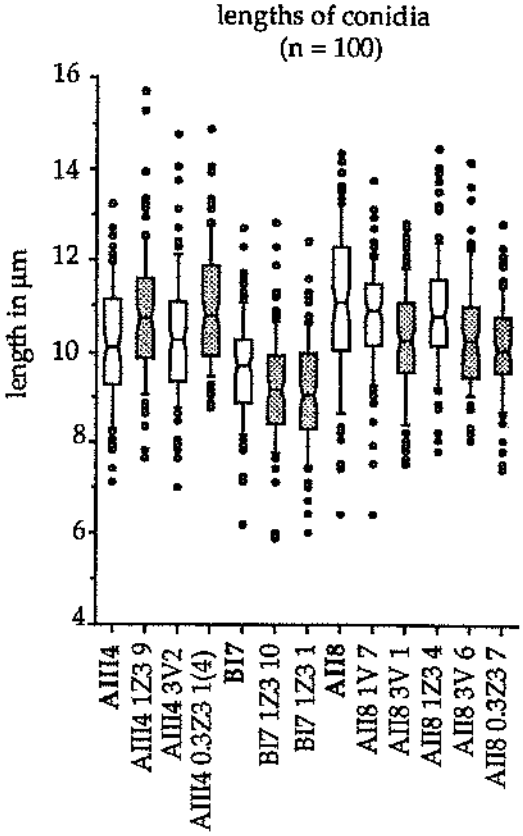


Competition test

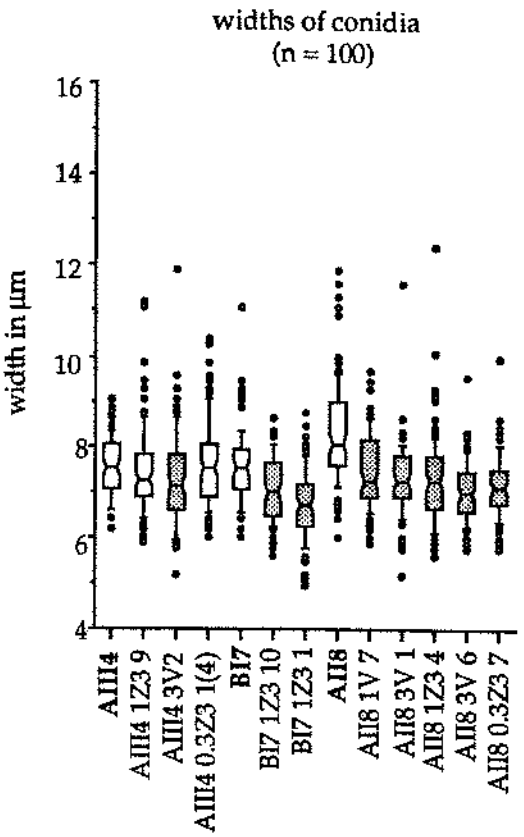
The laboratory resistant strains tested were not as competitive as their sensitive parental strains. As shown in Fig. 3.13 in the first generation the proportion of laboratory resistant conidia decreased. From the second to the fourth generation the proportion of resistant conidia became approximately stabilized at a low level of resistant conidia. Although the competitive ability of laboratory resistant strains was low compared to the sensitive parental strains, they did not disappear totally. In contrast to the laboratory resistant strains the dicarboximide field resistant strain (G 6.15 1.6) was more competitive than its sensitive competitor (G 6.15 1.8) from the same ascus (Fig. 3.13). This mixture, however, became contaminated after the 3rd generation and the dynamics in the population could not be followed further.

The strains included in the competition test were further used to study the influence of the acquisition of laboratory resistance on other fitness parameters. Fig. 3.14 shows the mean daily increase

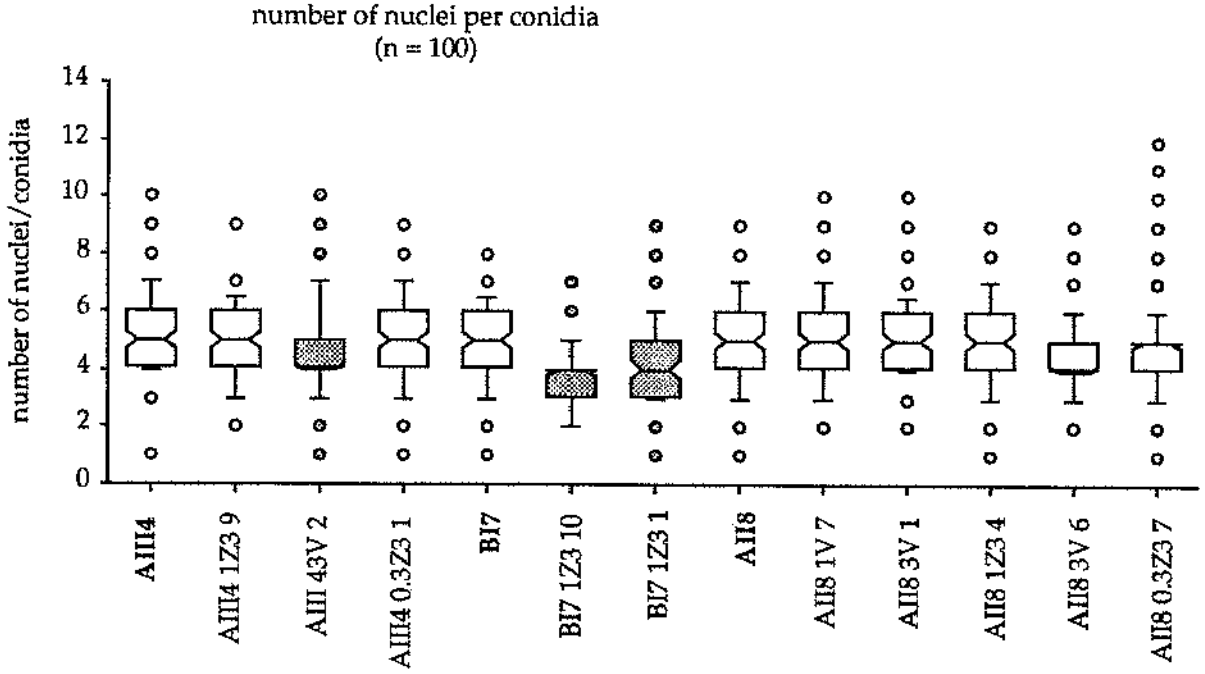
Fig. 3.12: Notched box plots of the frequency distribution of lengths, widths and number of nuclei per conidium of laboratory resistant strains and their sensitive parental wild type strains. Shadowed box plots indicate significant differences among the parental strains and their laboratory resistant strains (t-test, $p < 0.05$)



parental strains **(bold)** followed by their laboratory resistant strains



parental strains **(bold)** followed by their laboratory resistant strains



parental strains **(bold)** followed by their laboratory resistant strains

in colony diameter over 3 days. 5 from 8 laboratory resistant strains showed a significantly lower increase in colony diameter than their sensitive parental strains (Mann Whitney U test, $p<0.05$). The field resistant strain grew as fast as the sensitive strain from the same ascus.

In the pathogenicity assay all the resistant strains were significantly less virulent than their sensitive parental strains or the sensitive strain from the same ascus. Fig. 3.14 shows the mean lesion diameters assessed on Golden Delicious. Although the differences in the mean lesion diameters were significant between the field resistant strain and the sensitive strain from the same ascus and between the laboratory resistant strain CII5 0.3Z3 2 and its sensitive parental strain CII5, these resistant strains showed a higher virulence than the other laboratory resistant strains.

Fig. 3.13: Changes in the proportion of resistant and sensitive conidia during transfer experiments. Resistant and sensitive strains were mixed 1:2.

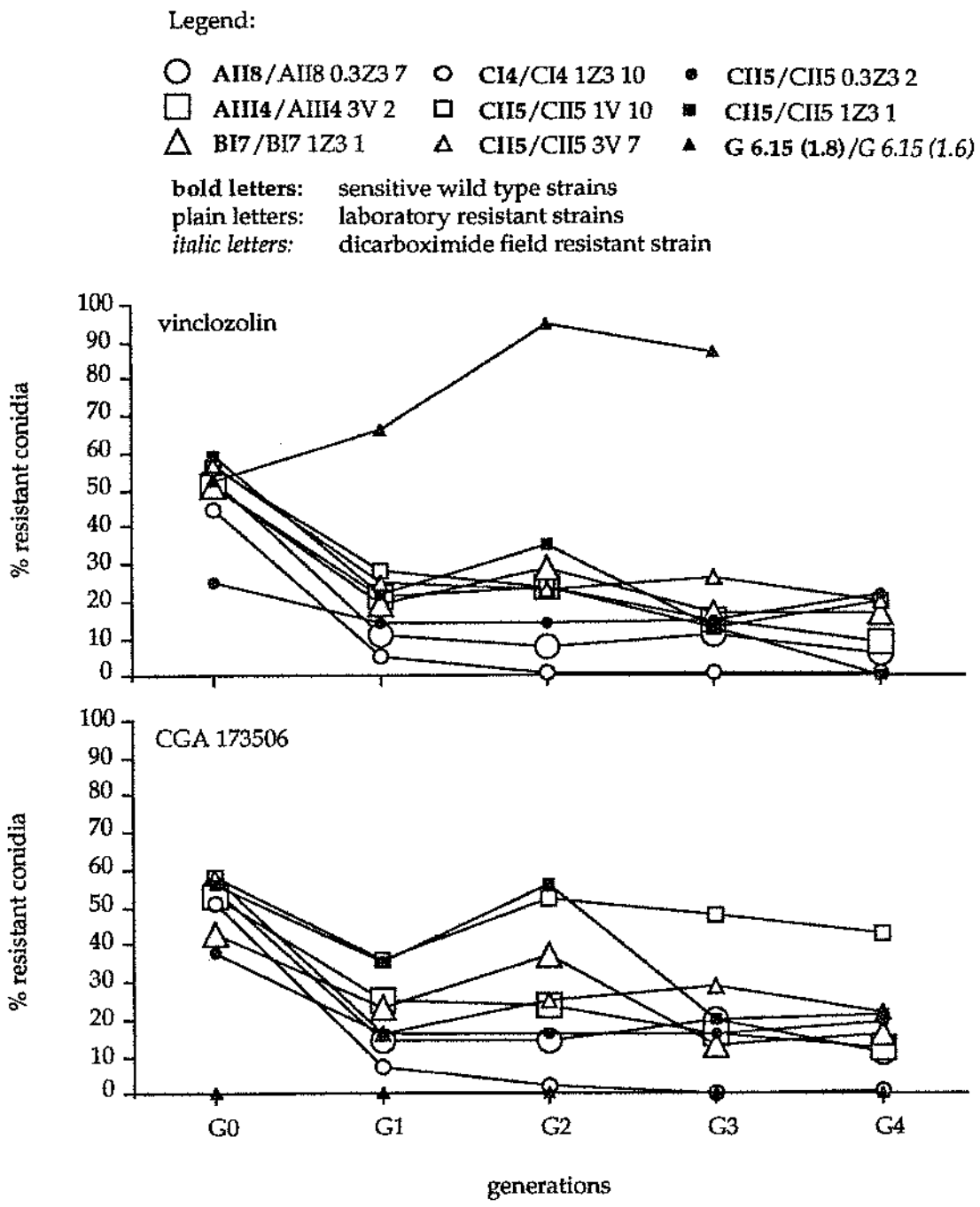
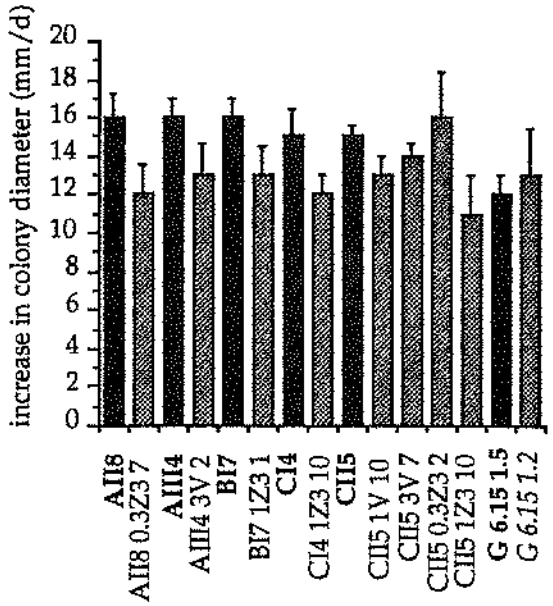
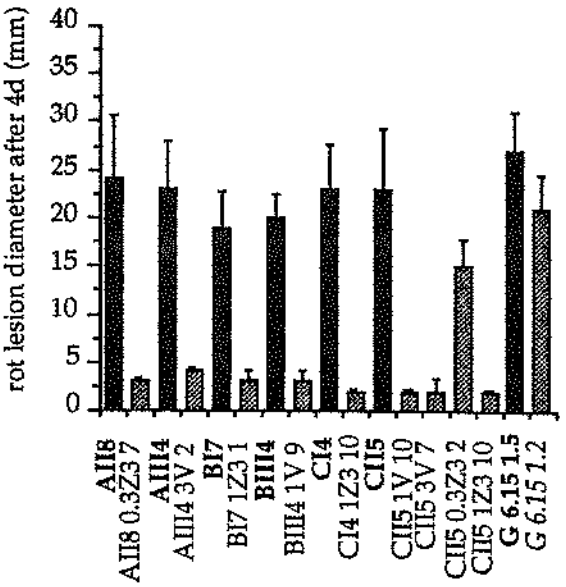


Fig. 3.14: Mean daily increase in colony growth and virulence on apple of strains used in the competition experiment (parental strains: black bars; laboratory resistant strains: shaded bars).



parental strains **(bold)** followed by their laboratory resistant strains or by the field resistant strain *(italic)* in alphabetic order



parental strains **(bold)** followed by their laboratory resistant strains or by the field resistant strain *(italic)* in alphabetic order

Stability of resistance

The EC50 values for vinclozolin of 10 sensitive wild type strains were significantly higher (t-test, $p < 0.05$) after 10 subcultures on pea agar. However, the resistance pattern of strains AI4 to CII5 (Table 3.5) to vinclozolin and to CGA 173506 did not change after 10 subcultures on pea agar (Table 3.5).

Six strains (BI7 1V 1, BI7 3V 3, BII1 1V 3, BIII4 0.3Z3 3, BIII4 1V 2, CII5 3V 1) that were derived from sensitive wild type strains on agar amended with various amounts of CGA 173506 or vinclozolin, respectively, but did not show decreased sensitivity to either active ingredient were transferred on apples. Five of these six strains were likewise subcultured on pea agar medium. All strains kept their sensitivity during the subculturing and the transfers on apples (Table 3.5).

Of 16 highly laboratory resistant strains (AI4 1Z3 6, AII8 0.3Z3 7, AII8 1V 7, AII8 1Z3 4, AIII4 0.3Z3 1(4), AIII4 1Z3 9, AIII4 3V 2, BI7 1Z3 1, BI7 1Z3 10, BII1 0.3Z3 1, BII2 1V 6, BII2 1Z3 7, BIII4 3V 6, CI4 1Z3 10, CII5 0.3Z3 2 and CII5 3V 7), all maintained their resistance to both fungicides during 10 subcultures on pea agar. From 9 highly laboratory resistant strains, 6 remained unchanged (AII8 3V 1, AIII4 0.3Z3 1(4), AIII4 3V 2, BII2 1V 6, CI4 1Z3 10 and CII5 3V 7) concerning their resistance level, whereas two strains (AIII4 1Z3 9 and CII5 1Z3 8) became sensitive to both fungicides when transferred on apple three times; one strain (AII8 1V 7) further tolerated high concentrations of vinclozolin but lost the ability to grow on agar containing high concentrations of CGA 173506 (Table 3.5).

Stability of resistance was tested for five low laboratory resistant strains (BIII4 0.3Z3 5, BIII4 1V 9, BIII4 3V 10, CII5 1Z3 1 and CII5 3V 6) by transferring them on apple and for 6 low laboratory resistant strains (BIII4 0.3Z3 5, BIII4 1V 9, BIII4 3V 10, CII5 0.3Z3 5, CII5 1V 10 and CII5 3V 6) by sub-culturing them on pea agar. From the five strains trans-

Table 3.5: EC50 values in mg/L (mycelial growth test)

	before 1st transfer		after 3rd transfer on apple		after 10th passage on pea agar	
strains	vinclozolin	CGA 173506	vinclozolin	CGA 173506	vinclozolin	CGA 173506
AI4	0.19	0.012	---	---	0.52	0.0017
AI7	0.15	0.0027	---	---	0.37	0.0028
AII8	0.18	0.0076	---	---	0.82	0.0091
AIII4	0.20	0.0079	---	---	0.57	0.0067
BI7	0.21	0.0066	---	---	0.47	0.0066
BII1	0.13	0.0030	---	---	0.23	0.0026
BII2	0.19	0.012	---	---	0.55	0.0087
BIII4	0.20	0.0083	---	---	0.55	0.011
CI4	0.14	0.0070	---	---	0.22	0.0034
CII5	0.22	0.016	---	---	0.67	0.020
AI4 1Z3 6	>100	0.69	---	---	>100	1.2
AI7 1V 8	3.2	0.023	0.53	0.0046	---	---
AII8 0.3Z3 7	>100	>10	---	---	>100	>10
AII8 1V 7	>100	0.39	>100	0.24	>100	0.83
AII8 1Z3 4	>100	2.9	---	---	>100	>10
AII8 3V 1	>100	2.1	>100	1.5	---	---
AII8 3V 6	2.0	0.020	2.2	0.024	3.7	0.044
AIII4 0.3Z3 1(4)	>100	>10	>100	>10	>100	>10
AIII4 1Z3 9	>100	0.92	0.52	0.0064	>100	1.9
AIII4 3V 2	>100	>10	>100	>10	>100	>10
BI7 1V 1	0.26	0.0057	0.48	0.0044	---	---
BI7 1V 2	1.4	0.025	6.3	0.027	2.7	0.025
BI7 1Z3 1	>100	>10	---	---	>100	>10
BI7 1Z3 10	>100	0.50	---	---	>100	0.95
BI7 3V 3	0.26	0.0086	0.44	0.0059	0.63	0.0075
BII1 0.3Z3 6	>100	0.25	>100	0.11	---	0.0038
BII1 1V 3	0.19	0.0066	0.47	0.0028	0.22	0.0026
BII2 0.3Z3 1	>100	2.6	---	---	75.0	>10
BII2 1V 6	>100	6.3	33.8	3	>100	>10
BII2 1Z3 7	>100	5.3	---	---	>100	>10
BIII4 0.3Z3 3	0.21	0.0060	0.55	0.0078	0.64	0.016
BIII4 0.3Z3 5	6.8	0.075	0.56	0.013	>100	0.16
BIII4 1V 2	0.37	0.0081	0.57	0.0078	0.65	0.015
BIII4 1V 9	6.3	0.095	5.1	0.083	6.4	0.14
BIII4 3V 10	6.3	0.078	7.3	0.061	7.4	0.16
BIII4 3V 6	>100	0.75	---	---	>100	2.2
BIII4 3V 7	0.62	0.021	---	---	1.4	0.027
CI4 1Z3 10	>100	>10	>100	>10	61.0	>10
CII5 0.3Z3 2	>100	>10	---	---	>100	>10
CII5 0.3Z3 5	7.2	0.25	---	---	>100	0.40
CII5 1V 10	5.6	0.17	---	---	87.0	0.27
CII5 1Z3 1	7.6	0.26	>100	0.27	---	---
CII5 1Z3 8	>100	0.92	0.58	0.0046	---	---
CII5 3V 1	0.34	0.015	0.55	0.010	0.60	0.021
CII5 3V 6	7.3	0.23	4.9	0.028	23.0	0.29
CII5 3V 7	>100	0.38	>100	0.44	>100	0.63

Table 3.5 (page 44):
EC50 values of sensitive and vinclozolin and CGA 173506 laboratory resistant strains prior and after subculturing on apples or on pea agar, respectively.
Classification of resistance patterns (based on results given in chapter 4):
CGA 173506 sensitive (CS) (EC50 < 0.03 mg CGA 173506/L), CGA 173506 low resistant (CLR) (0.03 mg CGA 173506 < EC50 < 0.3 mg CGA 173506/L), CGA 173506 highly resistant (CHR) EC50 > (0.3 mg CGA 173506/L)
vinclozolin sensitive (VS) (EC50 < 1 mg vinclozolin/L), vinclozolin low resistant (VLR) (1 mg vinclozolin < EC50 < 30 mg vinclozolin/L), vinclozolin highly resistant (VHR) EC50 > (30 mg vinclozolin/L)

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ferred on apple 2 maintained their resistance (BIII4 1V 9 and BIII4 3V 10), whereas 1 strain became sensitive (BIII4 0.3Z3 5) and 2 strains behaved intermediate (CII5 1Z3 1 and CII5 3V 6). Three of 6 strains subcultured on pea agar maintained their low laboratory resistance (BIII4 1V 9, BIII4 3V 10 and CII5 3V 6), while two strains kept their low laboratory resistance to CGA 173506 but increased their resistance to vinclozolin (BIII4 0.3Z3 5 and CII5 1V 10) and 1 strain (CII5 0.3Z3 5) became highly resistant to both active ingredients (Table 3.5).

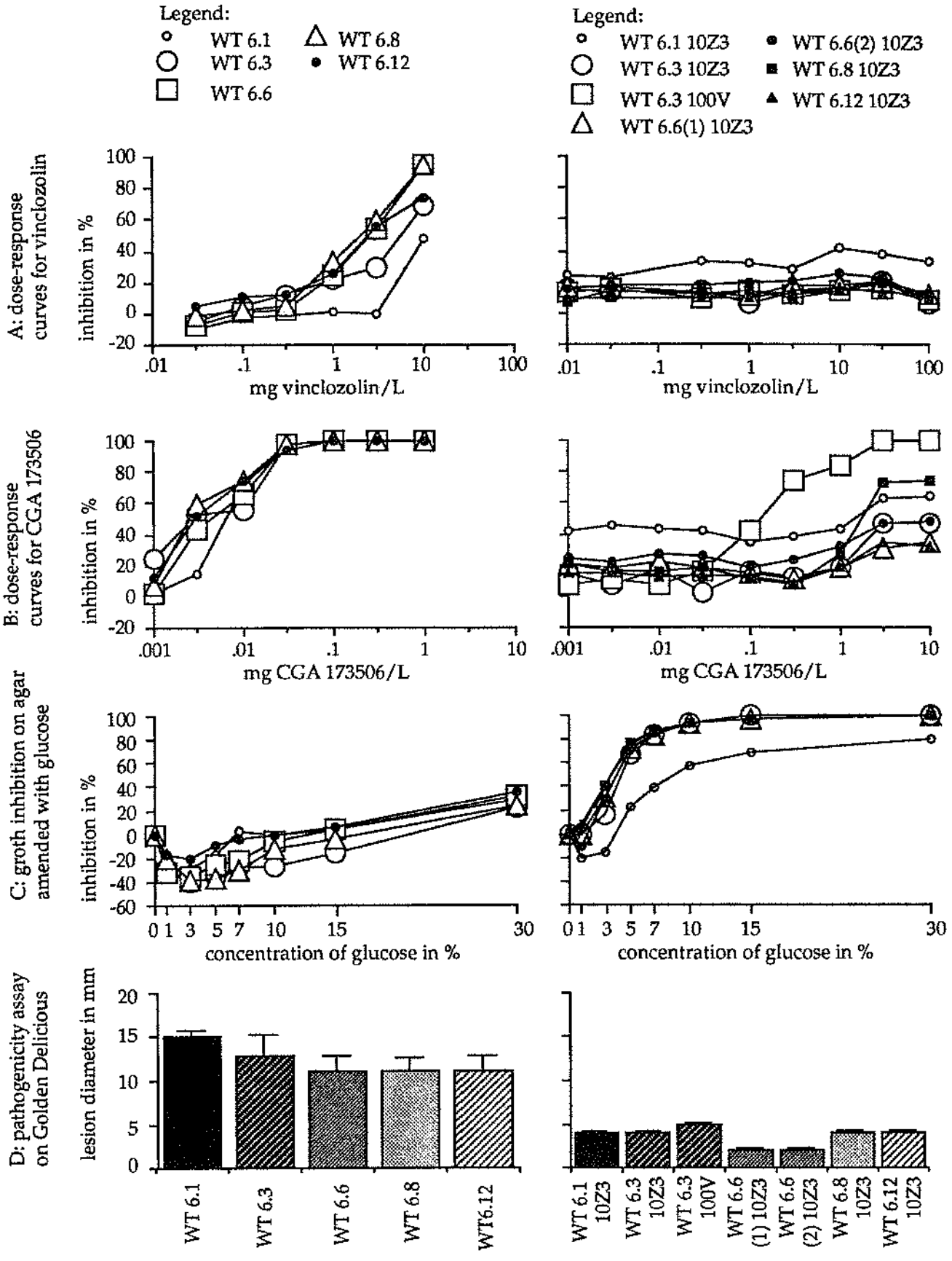
As it was expected for the multinucleate, heterokaryotic *B. fuckeliana*, intermediate forms occurred. It has thus to be considered that the threshold levels separating sensitive, low and highly laboratory resistant strains were defined arbitrarily and the transitions between the resistance patterns are smooth. Values of EC50 for four intermediate strains (AI7 1V 8, AII8 3V 6, BI7 1V 2 and BII1 0.3Z3 6), three of which low laboratory resistant to vinclozolin but sensitive to CGA 173506 (AI7 1V 8, AII8 3V 6 and BI7 1V 2) and one highly laboratory resistant to vinclozolin and low laboratory resistant to CGA 173506 (BII1 0.3Z3 6) were compared after subculturing on pea agar or on apples, respectively, with the original EC50 values. After 3 transfers on apple the strain which was highly laboratory resistant to vinclozolin and low laboratory resistant to CGA 173506 (BII1 0.3Z3 6) maintained its atypical resistance pattern. One of the 3 vinclozolin low laboratory resistant, CGA 173506 sensitive strains became sensitive to both active ingredients (AI7 1V 8), whereas two maintained their low laboratory resistance to vinclozolin after three transfers on apple (BI7 1V 2 and BIII4 3V 7). During the subculturing on pea agar, one strain kept its uncharacteristic resistance pattern (BI1 1V 2) while in another strain (AII8 3V 6) the sensitivity to CGA 173506 decreased to the level of low laboratory resistance and one strain was not tested (Table 3.5).

Fitness of laboratory resistant strains as compared to their vinclozolin field resistant parental strains

Fig. 3.15. A and B show the dose-response curves for vinclozolin and CGA 173506, respectively, of field resistant strains and of laboratory resistant strains, derived from these field resistant strains. The field resistant strains showed low resistance to vinclozolin (0.3mg vinclozolin/L < EC50 < 30 mg vinclozolin/L) while they were sensitive to CGA 173506 (EC50 < 0.03mg CGA 173506/L) (Fig. 3.15A and B left charts). The laboratory resistant strains, however, were highly resistant to vinclozolin (Fig. 3.15 A right chart) and they showed, in contrast to the field resistant strains, a decreased sensitivity to CGA 173506. Six out of seven strains were highly resistant to CGA 173506 whereas one strain showed low resistance (Fig. 3.15B right chart).

On media amended with up to 30% glucose, the field resistant strains were inhibited by less than 50%, compared to the control which grew on glucose free media. Mycelial growth increased on agar amended with low concentrations of glucose (Fig.

Fig. 3.15 A-D: Dose-response curves of vinclozolin and CGA 173506, osmotolerance and virulence of vinclozolin and CGA 173506 laboratory resistant strains and their vinclozolin field resistant parental strains



3.15C left chart). In contrast to the response of vinclozolin field resistant strains the mycelial growth of laboratory resistant strains decreased drastically when the osmotic pressure was increased (Fig. 3.15C right chart). Five laboratory resistant strains were completely inhibited and one strain was inhibited by more than 80% on agar amended with 30% glucose (Fig. 3.15C right chart). Strain WT 6.3 100V showed morphologically abnormal colony growth on media amended with glucose; its response to high osmotic pressure could therefore not be investigated.

In the pathogenicity assay all the laboratory resistant strains were less virulent than their sensitive parental field resistant strains.

The laboratory resistant strains differed from their vinclozolin field resistant parental strains in sensitivity to vinclozolin and to CGA 173506 as well as in osmotolerance and virulence. In contrast to the laboratory resistant strains their field resistant parental strains tolerated higher concentrations of glucose in the medium and were more virulent on apple. The acquisition of laboratory resistance was thus related to a loss of fitness in all cases.

3.4 Discussion

The ease of selecting laboratory resistant strains without treatments of UV irradiation or other mutagenic agents was described by various authors for dicarboximide fungicides (Leroux et al., 1977, Schüepp and Küng, 1978, Hisada et al. 1979, Maraite et al. 1980, Schüepp et al., 1982, Martinetti, 1986 and Wang et al. 1986). They found fast growing sectors which developed from sensitive strains on agar amended with lethal or strongly inhibiting concentrations of the fungicides. Hisada et al. (1979) and Martinetti (1986) also found colonies emerging from conidia on agar amended with either procymidone or vinclozolin in high concentrations. From their experience with methyl-benzimidazolecarbamates and dicarboximides, respectively, Bolton (1976) and Leroux et al. (1977) considered resistant strains to be already existent before the introduction of a new chemical. Results of experiments with CGA 173506 supported this hypothesis. From ten sensitive wild type strains of *B. fuckeliana* laboratory resistant strains could be selected with the same ease on media amended with lethal concentrations of CGA 173506 as it was previously demonstrated for dicarboximide fungicides. In contrast to Lorenz and Eichhorn (1982) who reported dicarboximide resistance to be a classical case of adaptive (= physiological) resistance, Hisada et al. (1979), Davis and Dennis (1981) and Ruiz Borge (1988) concluded from their results the resistance to be constitutional (= genetically fixed). In an adaptation test including CGA 173506 and vinclozolin there was no change in sensitivity neither to the phenylpyrrole nor to the dicarboximide fungicide after 5 transfers on agar amended with sublethal concentrations of both active ingredients, which indicated that resistance to CGA 173506 as well as to vinclozolin was not adaptive.

In the literature, the occurrence of laboratory resistant strains has been described as sectorial growth. This is, however, an over-simplification as the "runaways" do not necessarily have to emerge in the classical form of a sector. Schüepp and Küng (1978) showed that fast growing sectors either developed from slowly growing colonies or from totally inhibited colonies, respectively. In the binary classification system used here, these two types corresponded to growing type I and III, respectively. Although the results were not clear cut, it was obvious that "runaways" of type IA and IB mainly were highly resistant to vinclozolin and CGA 173506, respectively, while "runaways" of type IIIE and IC mostly did not show a reduced sensitivity to either of the two active ingredients. Even though it was not possible to derive their resistance pattern from the morphology of the "runaways" in all cases, it was, however, surprising that there was a clear evidence

for the connection of high laboratory resistance to vinclozolin and CGA 173506, respectively, and the morphological appearance of type IA and IB.

Dicarboximide field resistant *B. fuckeliana* strains differ from laboratory resistant strains with respect to their sensitivity to CGA 173506. While the former are sensitive to CGA 173506 (Gehmann et al., 1990) the laboratory resistant strains show a reduced sensitivity also towards CGA 173506. Similarly Martinetti (1986) found no cross resistance to the phenylpyrrole fungicide fenpiclonil in vinclozolin field resistant strains but she found reduced sensitivity to both active ingredients when working with laboratory induced mutants. The close association of dicarboximide and phenylpyrrole resistance in laboratory resistant strains might be interpreted as cross resistance between the two groups of fungicides, however, the high CGA 173506 sensitivity of dicarboximide field resistant isolates is contradictory to this hypothesis. Faretra and Pollastro (1991) showed in sexual crosses the segregation of vinclozolin field resistance (= low resistance) and laboratory resistance (= high resistance) in the ratio one to one, which indicates that low resistant and highly resistant phenotypes are caused by mutations in the same gene. Therefore it can be concluded that dicarboximide laboratory and dicarboximide field resistance are due to different alleles of the same gene.

While Leroux (1991) assumed the same mode of action for vinclozolin and fenpiclonil Martinetti (1986) proposed the existence of two different modes of action for fenpiclonil and vinclozolin and two different modes of resistance for field and laboratory resistance to vinclozolin which was later disproved by Faretra and Pollastro (1991). To elucidate the connection of modes of action and modes or resistance in phenylpyrroles and dicarboximides more data on the genetic basis of resistance to these active compounds are needed.

Under laboratory conditions resistant strains with a reduced fitness could survive whereas under field conditions they became suppressed. Vinclozolin field resistant strains were all of low resistance. As these strains could easily be detected in the field they were competitive to a certain extent. In the laboratory their high fitness was confirmed by their osmotolerance and their comparably high virulence on apple. Vinclozolin and CGA 173506 double resistant strains were never found in the field but they were readily induced under laboratory conditions.

Katan (1985) and Wang et al. (1986) produced laboratory resistant strains from vinclozolin field resistant strains. The present studies showed that laboratory resistant strains that occurred from vinclozolin field resistant strains were highly resistant to vinclozolin and to CGA 173506 but lost their fitness and thus were not able to survive in nature. These results provided an explanation for the lack of double resistant strains in nature but not for the absence of highly vinclozolin resistant or CGA 173506 resistant strains. It was, however, assumed that the fitness of highly vinclozolin resistant strains was seriously affected, which leads to a suppression of these strains under field conditions whereas strains that were resistant to only CGA 173506 were lethal as they could not even be found under the favorable conditions in the laboratory.

The comparison of mycelial growth rates of vinclozolin and CGA 173506 laboratory resistant strains and their sensitive parental strains reflected the contradictory results given in the literature. Pommer and Lorenz (1985) and Ruiz Borge (1988) only found slight differences in mycelial growth between sensitive and dicarboximide resistant strains whereas Davis and Dennis (1981) found growth rates of dicarboximide laboratory and field resistant strains to be inferior to sensitive strains. Wang et al. (1986) found minute differences in colony growth between dicarboximide field resistant, low laboratory resistant and

sensitive strains but they found considerable differences in growth when comparing highly laboratory resistant strains to sensitive strains.

The infective ability of *B. fuckeliana* is apparently dependent on the host. Davis and Dennis (1981) found no difference in the infective ability of dicarboximide resistant strains when tested on strawberries or tomato leaves. Corresponding results were reported by Wang et al. (1986) for cotyledons of cucumbers and from Lorenz and Eichhorn (1978) for leaves of Vicia faba. On the contrary, laboratory resistant strains were less virulent than sensitive strains when tested on carrots (Davis and Dennis, 1981). Leroux et al. (1977) working with cucumbers and Schüepp and Küng (1978) working with apples found some laboratory resistant strains to be as virulent as sensitive strains but, in accordance to Pommer and Lorenz (1985), they too found laboratory resistant strains that lost their pathogenicity. The present studies on the infective ability of 34 strains which were either sensitive or low to highly laboratory resistant to CGA 173505 and vinclozolin confirmed a generally decreased virulence of laboratory resistant strains. Vinclozolin laboratory resistant strains have been reported to be poor competitors with sensitive types and have frequently shown a tendency to decline in or disappear from mixed populations (Hisada et al., 1979, Gullino and Garibaldi, 1981, Pommer and Lorenz, 1985, Wang and Coley-Smith, 1986, Ruiz-Borge, 1988). In a competition test in absence of selection pressure CGA 173506 laboratory resistant strains behaved as described above for vinclozolin resistant strains.

In mycelial cells of *B. fuckeliana* Menzinger (1965) found 3 to 120 nuclei whereas in conidia he found 3 to 15 nuclei. Hansen and Smith (1932) suggested that the basic unit of an individual is the nucleus and not the cell; a multinucleate spore then represents a colony which will only give rise to a genetically pure culture if all the nuclei are genetically identical. In accordance with the results presented here for vinclozolin and CGA 173506 laboratory induced resistant strains, Lorenz and Eichhorn (1982), who investigated vinclozolin field resistant strains, did not find differences in nuclear numbers among resistant and sensitive strains. However, Hansen and Smith (1932), Lauber (1973) and Shirane et al. (1988), in contrast to Menzinger (1965), found a positive correlation between conidial dimension and number of nuclei, and Phillips et al. (1987) reported a positive correlation of increased glucose concentration in the growth medium and increase in volume, nuclear number and aggressiveness of the spores produced on the medium.

Menzinger (1965) underlined the importance of the heterokaryotic state of *B. fuckeliana* and the formation of anastomoses with regard to the enormous variability in this fungus. This high variability is of practical relevance as it allows the rapid development of fungicide resistance leading to serious problems in chemical control (Lorenz, 1983). However, various factors influence the fast buildup of a resistant population. Resistant strains have to be as competitive and as virulent as sensitive strains and the resistance must be stable. For strains resistant to methyl-benzimidazolecarbamate fungicides both assumptions are true (Bolton, 1976, Schüepp, 1981, Pommer and Lorenz, 1982). In the dicarboximides the situation is different, in practice populations of *B. fuckeliana* may show extreme fluctuations in sensitivity from year to year (Schwinn and Morton, 1990). Under laboratory conditions, however, laboratory resistance to vinclozolin and to CGA 173506 was generally stable although in some cases resistance was lost after transfers in the absence of a selection pressure. For vinclozolin these results were confirmed by Davis and Dennis (1981), Lorenzini (1983), Wang and Coley-Smith (1986) and Ruiz-Borge (1988).

Heterokaryosis provides a mechanism to maintain nuclei carrying different genetic information, e.g., for resistance or sensitivity, respectively, in the same cell or hyphal strand (Summers et al., 1984). In *B. fuckeliana* the (genetically different) nuclei can be exchanged between hyphal strands from different mycelia through anastomoses and within hyphal strands of the same mycelium through the septa between the hyphal cells. From crosses of diploid organisms the interplay of parental alleles leading to a uniform F1 generation is well known. Similarly it can be speculated about an interplay between different alleles from different haploid nuclei in multinucleate cells leading to a functional polyploidy. Selection pressure, e.g., exerted by a fungicide, may lead to the selection of cells (or hyphal strands) with a high proportion of nuclei that carry the suitable resistance gene. Esuruoso and Wood (1971) hypothesized that a sufficient number of nuclei carrying the gene for resistance must be present in hyphal strands or in spores of resistant fungal strains. They, however, proposed that spores and cells with too many nuclei which carry the information for resistance might lose their viability when the resistance gene has a negative pleiotropic effect on fitness. Prolonged and exclusive use of a fungicide group under high disease pressure can eventually lead to field resistance regardless of reduced fitness of resistant mutants (Staub and Sozzi, 1984). It can be hypothesized that in these heterokaryotic strains mutant and wild type alleles form an equilibrium in which the expression of resistance is caused by gene products of the mutant allele whereas the fitness of the fungus is influenced by the gene products from the wild type allele.

4. Study on the inheritance of resistance to CGA 173506 and vinclozolin and related fitness parameters in *Botryotinia fuckeliana* (de Bary) Whetzel

4.1 Introduction

Faretra and Antonacci (1987) and Faretra and Pollastro (1988) developed a reliable method to produce the sexual stage of *B. fuckeliana*. Faretra and Pollastro (1991) were able to elucidate the genetics of resistance to methylbenzimidazoles and dicarboximides, with special consideration of the effects of resistance genes on phenotypic characters relevant to crop protection. They analyzed random samples of 300 ascospores from each apothecium and thus could distinguish sensitive and resistant ascospores according to Leroux and Gredt (1981). Random spore analysis, however, did not allow distinguishment between progeny of different levels of resistance. In contrast to Faretra and Pollastro (1991) in the present investigations classical tetrad analysis was performed; 8 ascospores of single asci were cultivated and their colony growth on agar was assessed by using a wide range of fungicide concentrations. The inheritance of resistance to CGA 173506 and to vinclozolin was studied and the genetic basis of resistance against both active ingredients was compared.

Grindle (1983) showed the vinclozolin resistance gene vin-1 in *Neurospora crassa* Shear & Dodge to be allelic with the gene for osmotolerance os-1. Beever (1983) showed that all of 150 isolates, with one exception, that were resistant to dicarboximide in the field to be abnormally sensitive to high osmotic pressure. He raised the hypothesis that strains showing medium to high resistance to dicarboximides are always abnormally sensitive to osmotic pressure and thus will, because of their reduced fitness, occur infrequently in the field. The high number of nuclei per cell (Grindle, 1979, Lorenz and Eichhorn, 1982), the heterokaryosis (Davis, 1966) and the frequent formation of anastomoses (Lorenz, 1983) lead to the high genetic flexibility, which is a characteristic feature of heterokaryotic, asexually reproducing, field isolates of *B. fuckeliana* (Pommer and Lorenz, 1982). The instability and variability within progeny of individual heterokaryotic isolates might be caused by the selection and reassortment of genetically different nuclei during hyphal growth (Grindle, 1979).

In the present investigations the osmotic sensitivity and the pathogenicity of sexual progenies from sensitive strains, from strains carrying a laboratory-induced resistance to CGA 173506 and to vinclozolin, respectively, and from vinclozolin field resistant strains were compared.

4.2 Materials and Methods

Isolates were collected from strawberries and grapes at three locations in the Northeastern part of Switzerland (chapter 1, 2). Isolates resistant to vinclozolin in the field were collected from a vineyard that had been treated with dicarboximides for about 15 years (chapter 1). Laboratory induced resistant strains, which were resistant to vinclozolin and to CGA 173506, were derived from sensitive parental strains from strawberry fields which had never received any fungicidal treatments. They were obtained by subculturing from sectors spontaneously arising from the sensitive isolate incubated on malt agar amended with 1mg vinclozolin/L, 3mg vinclozolin/L, 0.3mg CGA 173506/L or 1mg CGA 173506/L, respectively (chapter 3).

Mycelium, sclerotia, microconidia and macroconidia from sensitive strains, from dicarboximide resistant field strains and from CGA 173506 and dicarboximide laboratory resistant strains were produced on malt agar (Oxoid No. 3). Sclerotia which developed

for 4 weeks at 15°C and for another 4 weeks at 0°C in the dark were picked with forceps from the agar and placed in distilled sterile water in test tubes. The sclerotia were then spermatized by adding a suspension of microconidia, macroconidia and mycelial fragments which were scraped from those plates from which the sclerotia were picked before (detailed procedure see Faretra and Pollastro (1988)). Apothecia were formed approximately 2 months after the spermatizing process. In the sexual crosses the microconidia which are uninucleate and apparently have only a sexual function (Grindle, 1979, Whetzel, 1929,1945) were used as male parental partners whereas sclerotia were used as the female partners. The role of macroconidia and mycelial fragments in the suspension that is used for spermatizing the sclerotia has not been investigated yet. Uninucleate microconidia which are produced from a homokaryotic strain of *B. fuckeliana* (which is very rare in nature) are all genetically identical whereas microconidia from a heterokaryotic strain (which is the typical case in asexually produced strains) are genetically different. Because of the frequent formation of anastomosis, between two different hyphae, the definition of an individual is difficult in this fungus. Therefore the use of microconidia from one (heterokaryotic) strain or from a couple of strains is in this case of minor importance.

Apothecia were crushed in 200µl of sterile distilled water on a cavity slide. Approximately 50µl of a single crushed apothecium in water were placed in the center of a malt agar plate. The plates with the fragments of the crushed apothecium, the liberated ascospores and the entire asci were exposed in a sterile cabinet to evaporate the excessive water. Asci either burst and released their ascospores or ascospores were liberated by breaking the ascus apart using a micromanipulator equipped with two fine glass needles. The 8 ascospores were separated and, after germination, they were transferred to new malt agar plates. These plates served as mother plates from which the single ascospore isolates were mass transferred to pea agar. After 5 to 10 days sporulating cultures were stored at 4°C in the dark.

Spore suspensions of 10^5conidia/ml were prepared from sporulating cultures and 50µl were added to agar discs of 10mm diameter. The agar discs were incubated at 20°C in the dark for 17h. They were then placed upside-down on fungicide amended agar and incubated at 20°C in the dark for 3 days. Vinclozolin was used in six concentrations for sensitive and field resistant strains, ranging from 0.03mg/L to 10mg/L and in eight concentrations for laboratory-resistant strains, ranging from 0.03mg/L to 100mg/L. CGA 173506 was used in six concentrations for sensitive and field resistant strains, ranging from 0.001mg/L to 0.3mg/L and in nine concentrations for laboratory-resistant strains, ranging from 0.001mg/L to 10mg/L. The mean diameters of colonies of 3 replicates were assessed and the percentage of inhibition of growth was calculated.

To assess osmotolerance, measured as glucose tolerance, malt agar discs, 10mm diameter, were inoculated with 50µl of a spore suspension of *B. fuckeliana* containing 10^5 conidia/ml and incubated for 17h at 20°C in the dark. The malt agar discs were placed upside-down on the glucose (sorbitol) agar medium (malt agar amended with 1, 3, 5, 7, 10, 15 and 30% glucose (or sorbitol)). The colony diameter of three replicates was measured after 3 days and inhibition of growth was calculated.

The pathogenicity assay described by Schüepp and Küng (1978) was slightly modified to assess the virulence of the single ascospore cultures (for details about the apple pathogenicity test see chapter 3.2, page 26).

Table 4.1: Characterization of strains used for the production of apothecia

Sclerotia of single strains were spermatized with mixtures of microconidia, macroconidia and mycelial fragments from various strains.

Sclerotia Partner	Origin	Resistance Pattern of Sclerotia Partner[a]		Spermatizing Partners[b]
Oe 4	strawberry, Wädenswil[c]	VS	CS	group I
G 6.7	grape, Walenstadt[c]	VS	CS	group II
G 6.9	grape, Walenstadt[c]	VS	CS	group II
WB II 5	grape leaf, Walenstadt[c]	VS	CS	group III
B 26.21	grape leaf, Walenstadt[c]	VLR	CS	group IV
G 6.11	grape, Walenstadt[c]	VLR	CS	group V
G 6.15	grape, Walenstadt[c]	VLR	CS	group V
AII8 3V 6	selected for resistance on 3mg vinclozolin/L [d]	VLR	CLR	group VI
AII8 3V 1	selected for resistance on 3mg vinclozolin/L [d]	VHR	CHR	group VII
AIII4 3V 2	selected for resistance on 3mg vinclozolin/L [d]	VHR	CHR	group VII
AII8 1Z3 4	selected for resistance on 1mg CGA 173506/L [d]	VHR	CHR	group VII

[a] Resistance pattern of sclerotia partner

VS	vinclozolin sensitive (EC50 < 1mg/L)	CS	CGA 173506 sensitive (EC50 < 0.03mg/L)
VLR	vinclozolin, low resistance (1mg/L < EC50 < 30mg/L)	CLR	CGA 173506, low resistance (0.03mg/L < EC50 < 0.3mg/L)
VHR	vinclozolin, high resistance (EC50 > 30mg/L)	CHR	CGA 173506, high resistance (EC50 > 0.3mg/L)

[b] spermatizing partners

group I	3 strains from strawberries, Wädenswil
group II	4 strains from grape leaves, Walenstadt
group III	12 strains from grapes and strawberries; various origins
group IV	3 strains from grape leaves, Walenstadt
group V	4 strains from grapes, Walenstadt
group VI	40 strains from strawberries, Wädenswil and Dietikon
group VII	7 strains from strawberries, Wädenswil and Dietikon

The strains of group I, VI and VII were all sensitive whereas those of group II, III, IV and V contained sensitive as well as strains with low resistance level to vinclozolin. None of these strains showed decreased sensitivity to CGA 173506.

[c] isolates from naturally infected fruits or leaves

[d] strains selected in the laboratory from isolates of strawberries from Wädenswil

4.3 Results

Table 4.1 shows the combinations of sclerotia partners and spermatizing partners which yielded in apothecia with ripe ascospores. The progeny of these ascospores was used to study the genetic variability with regard to fungicide resistance as well as virulence on Golden Delicious and osmotolerance. Sensitive strains, vinclozolin field resistant strains and strains of different levels of laboratory resistance to vinclozolin and CGA 173506 were selected as sclerotia partners. For the spermatizing step, strains were grouped.

Fig. 4.1 shows the reactions of progeny of eight spores from a single ascus to vinclozolin and CGA 173506. For vinclozolin, the classification of Faretra and Pollastro (1991) was slightly modified: EC50 of sensitive strains <1mg/L, EC50 of highly resistant strains is >30mg/L whereas EC50 values of strains with low resistance levels ranged from 1mg/L to 30mg/L. With regard to CGA 173506 strains were classified as sensitive strains with an EC50 < 0.03mg/L, as low resistant with an EC50 ranging from 0.03mg/L to 0.3mg/L and as highly resistant strains with an EC50 > 0.3mg/L.

The 120 single ascospore cultures tested, can all be assigned to four resistance patterns to vinclozolin and CGA 173506. Fig. 4.1 and Fig. 4.2 show data of representative asci for each of the four patterns. Concerning both ingredients, strains attributed to the first pattern were sensitive, strains ascribed to the second pattern were split into sensitive and highly resistant in the ratio one to one, whereas pattern three shows sensitive and low resistant strains in the ratio one to one. Single ascospore cultures which are sensitive to both ingredients resulted from the combination of sclerotia of the sensitive strain Oe4 with a mixture of three sensitive spermatizing strains (Fig. 4.1A). An equal splitting of single ascospore cultures in sensitive and highly resistant to vinclozolin as well as to CGA 173506 occurred by spermatizing strain AII8 1Z3 4 being highly laboratory resistant to vinclozolin and CGA 173506 with several sensitive strains (Fig. 4.1B). Pattern three reveals a genotype that is low resistant to both active ingredients. The ascospores tested originate from strain AII8 3V 6 which was low resistant to vinclozolin and to CGA 173506 and was spermatized with the same mixture of sensitive strains that was used to spermatize sclerotia of strain AII8 1Z3 4 (Fig. 4.1C).

In contrast to the results in Fig. 4.1 that were obtained from experiments with laboratory resistant strains, Fig. 4.2 shows representative data from 8 single ascospores of a single ascus of experiments with field resistant strains. Resistance pattern 4 (Fig. 4.2) shows single ascospore cultures that were obtained from a crossing of strain G 6.15, which was a vinclozolin field resistant strain. G6.15 was spermatized with microconidia from three sensitive strains and from one dicarboximide field resistant strain. Concerning vinclozolin the single ascospore cultures which originated from combinations of field resistant strains and sensitive strains reacted in the same way as those produced from laboratory resistant strains and sensitive strains. However, no resistance to CGA 173506 was found in progenies of strains which were field resistant to vinclozolin.

Results from tests on media supplemented with sorbitol, which has the same osmotic effect as glucose but cannot be metabolized by *B. fuckeliana* did not differ from results obtained from tests on media supplemented with glucose (data not shown).

Concerning osmotolerance and pathogenicity on apple, identical results were obtained from 48 single ascospore cultures of pattern 1 from 6 asci of different apothecia. Fig. 4.3A shows the data of a representative set of 8 ascospore isolates of a single ascus. These 8 strains showed a significant increase

Fig. 4.1: Pattern of genotype reaction to vinclozolin and CGA 173506 of progeny of eight ascospores from single asci

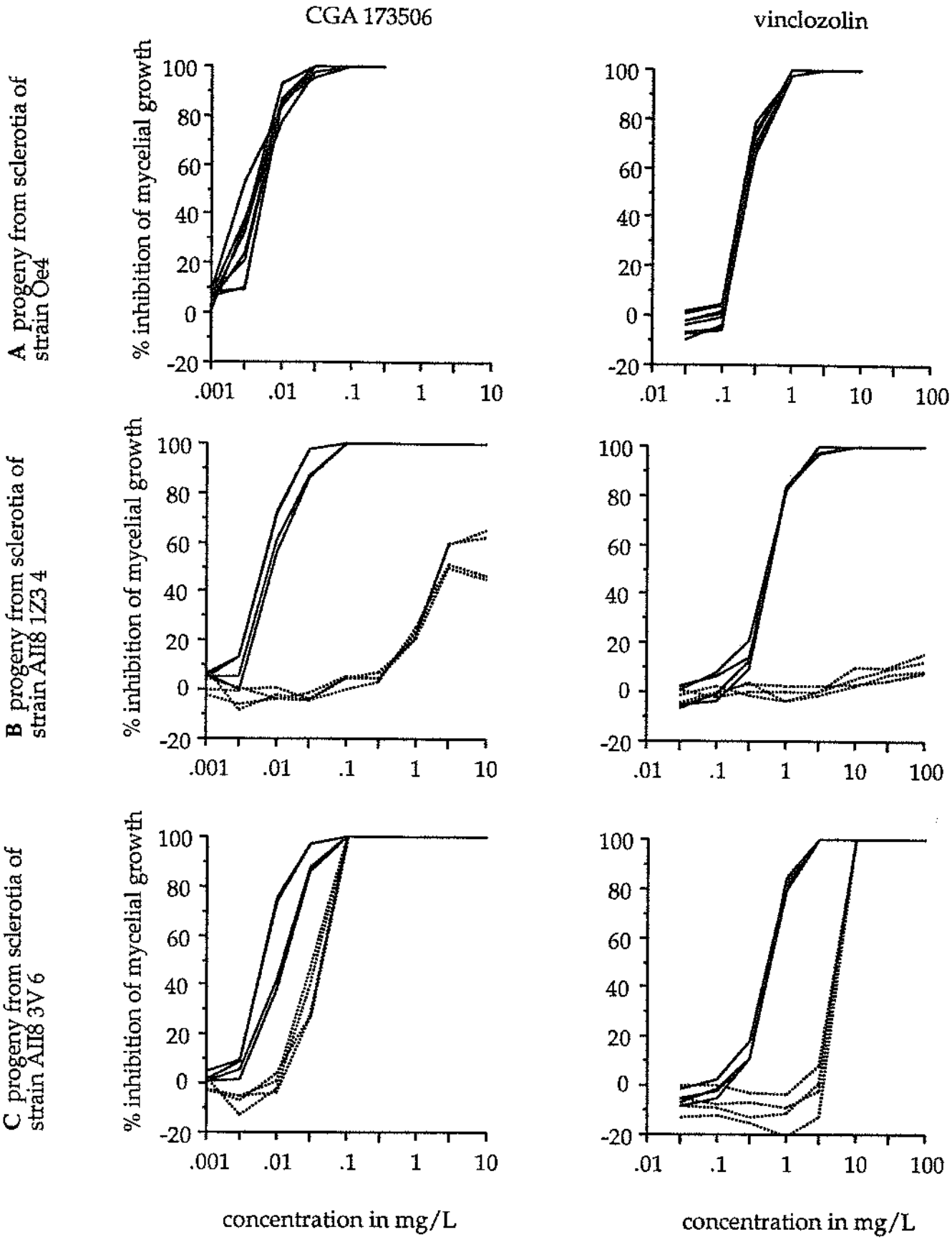
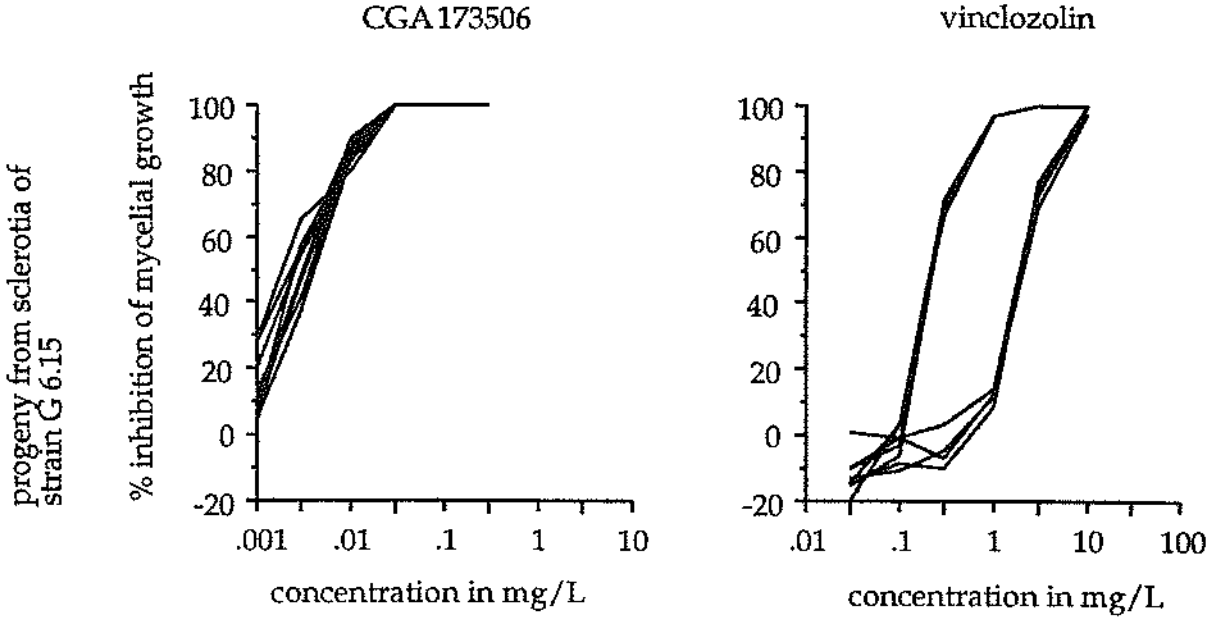


Fig. 4.2: Pattern of genotype reaction to vinclozolin and CGA 173506 of progeny of eight ascospores from single asci of dicarboximide field resistant sclerotia of strain G. 6.15



in colony diameter compared to the control on malt agar amended with up to 10% glucose (t-tests, $p<0.05$). Assays on Golden Delicious showed pathogenicity of all strains. Indeed, in a one factor analysis of variance, mean lesion diameters on apple differed significantly between different single ascospore cultures of the same ascus (F-test, $p=0.0001$). However, these differences are minute compared to the differences in lesion size between resistant and sensitive strains (Fig 4.3C,D).

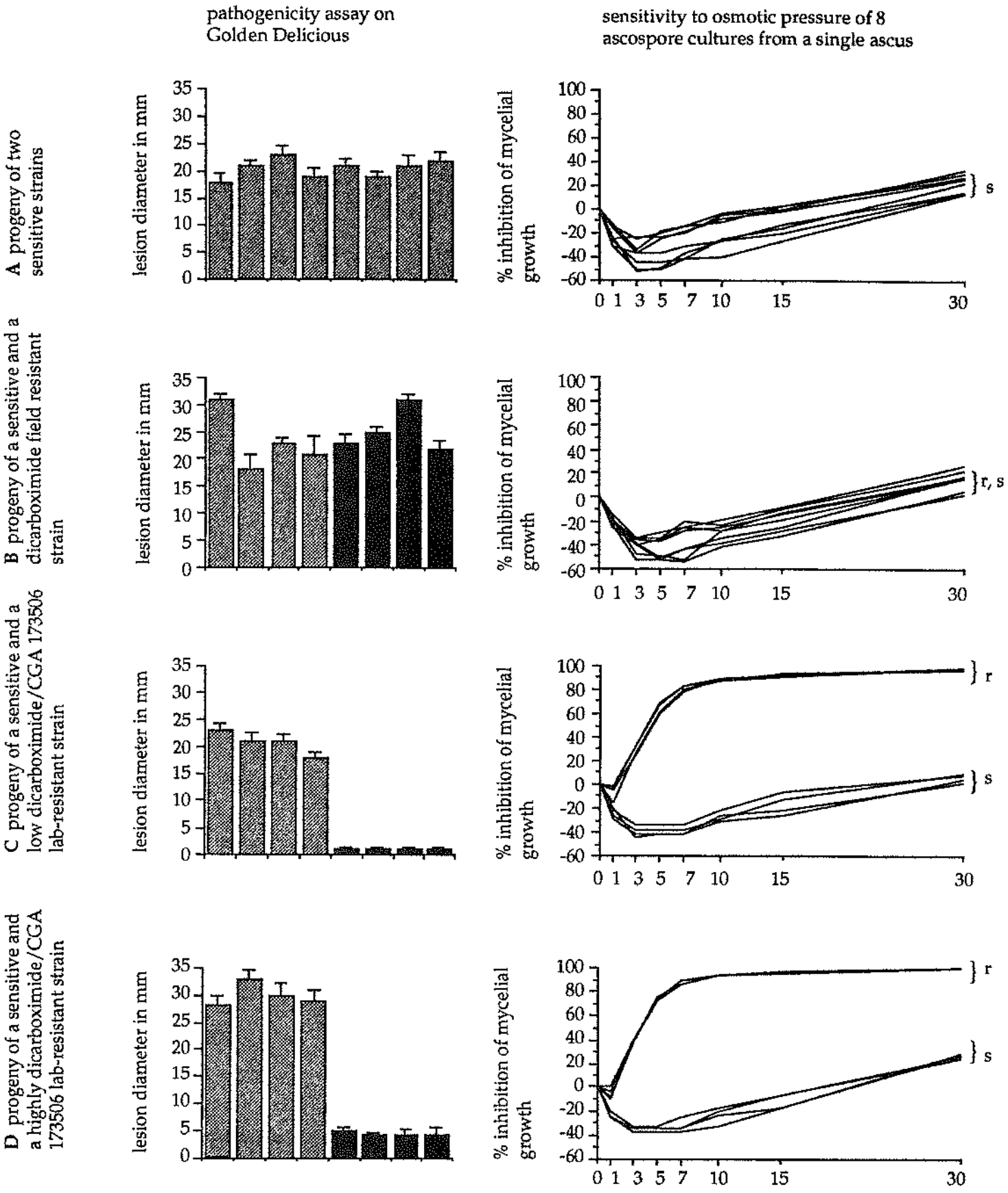
The results shown in Fig. 4.3B were obtained from an ascus of pattern 4. The 8 ascospore cultures were all pathogenic on Golden Delicious. The mean virulence of the four vinclozolin field resistant progenies and the four sensitive progenies, respectively, was not significantly different (t-test, $p=0.13$). The four field resistant isolates were tolerant to high osmotic pressure. However, compared to the four sensitive isolates the four vinclozolin low resistant isolates showed a small, but significant increase (t-tests, $p<0.05$) in osmotic sensitivity on agar amended with 3 to 10% glucose. Of the cultures that were obtained from two more asci of pattern 4 from different apothecia the single ascospore cultures showed the same virulence on apple as the 8 cultures which were derived from the ascus shown in Fig. 4.3B. The differences in osmotolerance were however minute and thus obscured by other phenotypic variation of these isolates (data not shown).

Seven asci of pattern 3 were obtained from 6 different apothecia. The characteristics of the 8 single ascospore cultures from a single ascus shown in Fig. 4.3C are representative for all the 7 asci that were tested, concerning increased glucose concentrations

Fig. 4.3: Pathogenicity assay on apple cv. Golden Delicious and sensitivity to high osmotic pressure (measured as glucose tolerance of 8 spores of single asci

(s) } sensitive single ascospore cultures

(r) } resistant single ascospore cultures



and the virulence on apples. As shown in Fig. 4.3C response to high osmotic pressure and pathogenicity of the 8 single ascospore cultures derived from a single ascus split in the ratio one to one. All the strains that showed decreased sensitivity to either vinclozolin or CGA 173506 showed increased sensitivity to high osmotic pressure and significantly decreased virulence or lack of pathogenicity on apple (t-test, $p=0.0001$). On agar amended with 1 to 30% glucose the four strains which showed a decreased sensitivity to both active ingredients differed significantly from the sensitive strains in the inhibition of mycelial growth (t-test, $p<0.05$). The four strains that were sensitive to vinclozolin and to CGA 173506 were pathogenic on Golden Delicious and they also tolerated high osmotic pressure.

Fig. 4.3D shows data of 8 single ascospore cultures which derived from a single ascus of pattern 2, that represented the progenies of 4 asci from 4 different apothecia. Pathogenicity on Golden Delicious and tolerance to high osmotic pressure split in the ratio 1:1. Resistant strains showed a significantly decreased virulence (t-test, $p= 0.001$) or even lack of pathogenicity. On media amended with increasing concentrations of glucose the sensitive strains behaved as described and explained in Fig. 4.3A, whereas the resistant strains behaved as described and explained in Fig. 4.3C.

4.4 Discussion

For the control of *B. fuckeliana* in grapes methylbenzimidazoles were withdrawn from the market in most vine growing areas because of the high genetic stability of the resistance and the high fitness of resistant strains in the field (Schüepp and Küng, 1981, Saracchi and Lorenzini, 1985). In contrast, in the case of the dicarboximides the generally lower fitness of resistant field strains still allows use in certain areas (Beever et al., 1991, Pak et al., 1990). The mode of action of the aforementioned botryticides has been clarified so far only for the methylbenzimidazoles (Davidse, 1988, Sisler, 1988). Studies on the genetics of resistance by Faretra and Pollastro (1991) showed that field isolates and laboratory mutants of *B. fuckeliana* can be classified into three phenotypes regarding their response to benomyl or to vinclozolin, respectively: sensitivity, low resistance and high resistance. The polymorphic major gene conferring resistance or sensitivity to the dicarboximide vinclozolin has been designed as Daf1, that to methylbenzimidazoles as Mbc1. Alleles coding for sensitivity, low resistance and high resistance have been assigned Daf1S, Daf1LR and Daf1HR for vinclozolin and Mbc1S, Mbc1LR and Mbc1HR for methylbenzimidazoles (Faretra and Pollastro, 1991). In the present investigation, genetic studies using classical tetrad analysis showed the same patterns of sensitivity or resistance, respectively, to phenylpyrrole as demonstrated by Faretra and Pollastro (1991) for dicarboximides and methylbenzimidazoles by random spore analysis. From this it was concluded that resistance to CGA 173506 was also located in a single gene. The major resistance gene responding to CGA 173506 is suggested to be designated as gene Pyr1 following Yoder et al. (1986). It was further hypothesized that three alleles (Pyr1S, Pyr1LR and Pyr1HR) are coding for sensitivity, low resistance and high resistance, respectively. This hypothesis, however, remains to be verified in future tests.

Homokaryotic dicarboximide resistant sexual progenies from crossings of heterokaryotic dicarboximide resistant field strains with heterokaryotic sensitive strains are weakly resistant to vinclozolin and they are sensitive to CGA 173506 (Fig. 4.2). Homokaryotic CGA 173506/ vinclozolin laboratory resistant sexual progenies from crossings of heterokaryotic CGA 173506/ vinclozolin laboratory resistant strains with heterokaryotic sensitive strains, show decreased sensitivity to vinclozolin and to CGA 173506 (Fig. 4.1). In contrast to the situation with methyl-benz-

imidazolecarbamates and dicarboximides, where Faretra and Pollastro (1991) demonstrated the independent segregation of the two major genes, the results reported here show cosegregation of Pyr1 and Daf1 (Fig. 4.1). This may lead to the following hypotheses:

1. The genetic basis of field resistance in *B. fuckeliana* is different for vinclozolin and for CGA 173506. However, it is identical in laboratory resistance for both active ingredients. This hypothesis provides an explanation for the lack of cross resistance between CGA 173506 and vinclozolin in the field and for the presence of cross resistance between the phenylpyrrole and dicarboximides in laboratory resistant strains. It is, however, contradicted by the results reported by Faretra and Pollastro (1991) who demonstrated dicarboximide field resistance (Daf1LR) and dicarboximide laboratory resistance (Daf1HR) to be caused by mutations in the same gene. They suggested that Daf1LR and Daf1HR are allelic.
2. Daf1 is different from Pyr1 and both genes are closely linked. This hypothesis explains the lack of cross resistance in the field but it is contradicted by the fact that independent segregation of Pyr1 and Daf1 was never found. The closer the linkage of the two genes the higher is the number of parental ditypes. The number of analyzed tetrads might then, however, have been too small to find the rarely occurring recombinant ditypes (= all the 8 sexual progenies within a single ascus are recombinants) and the tetratypes (= 4 of the 8 the sexual progenies within a single ascus are recombinants and 4 of them are identical with their parental strains).
3. Daf1 is identical with Pyr1 and the genetic basis of field and laboratory resistance is the same. In this case lack of independent segregation is obvious. The lack of cross resistance in dicarboximide resistant field strains can then be explained by an allele of Daf1 that only causes reduced sensitivity to vinclozolin but neither has an effect on the sensitivity to CGA 173506 nor has a pleiotropic effect on osmotolerance.

With respect to the use of the novel phenylpyrrole in the field a single locus for resistance to vinclozolin and to CGA 173506 is obviously more critical than two different loci. As the development of field resistance cannot be excluded, shifts in sensitivity of *B. fuckeliana* to the new fungicide in the field have to be carefully monitored. Past experiences with the dicarboximides and this particular pathogen, as well as these results, clearly indicate the need for a suitable anti-resistance strategy based on which the phenylpyrrole fungicide should be introduced into the market. Further studies on the resistance risk of CGA 173506 must focus on testing the above mentioned hypotheses. Sexual progenies from crosses of sensitive strains with CGA 173506/ vinclozolin laboratory resistant strains that were selected from dicarboximide resistant field strains or crosses between vinclozolin resistant field strains and CGA 173506/ vinclozolin laboratory resistant strains should be analyzed. Random spore analysis is, due to the possibility of testing a much bigger sample of ascospores, to prefer over classic tetrad analysis in this case where a close linkage is expected and recombinants are rare.

Mutants of *N. crassa* Shear & Dodge and of *Saccharomyces cerevisiae* Meyen ex Hansen which were hypersensitive to high osmotic pressure were characterized by Mays (1969) and Singh and Shermann (1978). Mays (1969) and Selitrennikoff et al. (1981) speculated that the products of the corresponding genes are involved in cell wall synthesis, i.e., in the supply of precursors for the cell wall synthesizing enzymes (Mays, 1969). This hypothesis was supported by the results of Livingston (1968, 1969) who found in osmotically sensitive mutants of *N. crassa* some differences in the overall cell wall composition as well as

in the cell wall gross appearance. A structural change in the membrane which affects cell wall synthesis may have occurred. Slight conformational disturbance of the cell wall synthesizing enzymes, or a change in the transport capabilities might cause such disturbance in cell wall metabolism and therefore lead to abnormal osmotic sensitivity.

Cosegregation of resistance to vinclozolin and CGA 173506, sensitivity to high osmotic pressure and pathogenicity suggest that sensitivity to high osmotic pressure and pathogenicity are pleiotropic effects of the resistance gene(s). Beever (1983), working with 20 laboratory-induced dicarboximide resistant, asexual strains of *B. fuckeliana* found 3 strains which were weakly resistant and did not show an increase in osmotic sensitivity. In contrast to Beever (1983) in the present investigations, all strains derived from single ascospore isolates of single asci, without exceptions, carried laboratory-induced resistance to dicarboximides and to CGA 173506 and showed an increase in sensitivity to high osmotic pressure and a decrease of virulence, independent of the level of resistance. The aforementioned genetic flexibility of *B. fuckeliana* might provide an explanation for the contradictory results. Mutations of only one of the nuclear components of a heterokaryon would be masked by the prevailing allele in the other components. The phenotype depends on the kind and proportion of the nuclei present in the heterokaryon (Esuruoso and Wood, 1971, Grindle, 1979).

Beever and Brien (1983) also tested 150 low level dicarboximide field resistant isolates of the pathogen. With only one exception, all were abnormally sensitive to high osmotic pressure. Sexual progenies from three crossings of low level dicarboximide field resistant strains with sensitive strains showed small, but significant differences in sensitivity to high osmotic pressure in one case. These results are in accordance with Faretra and Pollastro (1991) who usually found an increase in osmotic sensitivity of field resistant isolates when they compared them with sensitive strains. They found, however, the differences to be so small that they were easily obscured by other variations in the phenotypes. The virulence of low level dicarboximide field resistant strains was not reduced compared to the sensitive strains (Fig. 4.3B).

The finding of a connection between osmotic sensitivity and resistance against dicarboximides which was reported by various authors working with *N. crassa* and *B. fuckeliana* (Grindle and Temple, 1982, Beever, 1983, Beever and Brien, 1983, Grindle, 1983, Endlich and Lyr, 1987, Ellis et al., 1991) was also found in this investigation on *B. fuckeliana* resistant to CGA 173506. This leads to the question whether changes in cell wall synthesis and membrane permeability, which probably are involved in the increase of sensitivity to high osmotic pressure, are the cause of decreased sensitivity to dicarboximides and phenylpyrroles, respectively. The understanding of the biochemical basis of osmotic sensitivity in *B. fuckeliana* might help to elucidate the biochemical mode of action of dicarboximides and phenylpyrroles.

General Discussion

B. fuckeliana is a genetically highly variable organism with a worldwide distribution. Belonging to the most ubiquitous facultative plant pathogens it causes damage of great economic importance in grapes, small fruits, vegetables, bulbous monocotyledons, forest tree seedlings and glasshouse crops (Jarvis, 1977, Domsch et al., 1980, Coley-Smith, 1980, Mathys, 1982). The fact that *B. fuckeliana* is capable of attacking many diverse crops at almost any stage in their growth and in storage, and of affecting all plant parts, including the roots, cotyledons, leaves, stems, flowers and fruits makes it an extremely difficult fungus to control (Maude, 1980).

The response of fungal populations to fungicides used for their control, and hence the risk of disease control failure due to resistance depends on the biochemical mode of action of the active ingredient, on the extent of genetic variability available in the pathogen (Georgopoulos and Skylakakis, 1986) and the use pattern of the product. In *B. fuckeliana* the genetic flexibility is enormous due to its heterokaryotic state (Hansen and Smith, 1932, Hansen, 1942, Menzinger, 1965, Lauber, 1971, Grindle, 1979). By the formation of anastomoses, genetically different nuclei obtained by spontaneous mutation, can be freely exchanged in the fungal population. Due to this peculiarity in its biology *B. fuckeliana* is able, to a high degree, to adapt to changes in environmental conditions rapidly. For example laboratory resistant strains develop within a few days *in vitro*, when high selection pressure is exerted by the antifungal compounds fenpiclonil or vinclozolin (Leroux et al., 1977, Schüepp and Küng, 1978, Martinetti, 1986). In the field, high selection pressure exerted by the intensive and exclusive use of methyl-benzimidazolecarbamates and dicarboximides resulted in the development of field resistance and in loss of efficacy within a few years (Holz, 1979, Schüepp and Küng, 1981, Schüepp et al., 1982, Schüepp and Siegfried, 1983).

In the study presented here *B. fuckeliana* was chosen as a suitable model organism to investigate genetic variability and development of fungicide resistance for various reasons: its genuine genetic variability is vast, it is a fast growing, facultative pathogen that can be easily cultivated on artificial media, wild type isolates are abundant on a wide variety of host plants, sexual progenies are accessible for genetic studies, occurrence of resistance and damage caused by this organism is of economical importance. First reports on similarities in the mode of resistance between the new chemical group of phenylpyrroles (Gehmann et al. 1990) and the chemically unrelated group of dicarboximides (Martinetti, 1986, Leroux, 1991) were the starting point for the present investigations.

Repeated use of fungicides with the same mode of action leads to a shift of sensitivity of the target fungus population (Delp, 1988). Along with other measures restricted spray program, i. e., achieved by the use of less susceptible grape varieties that require less treatments has been advised to prevent or to delay the buildup of resistance in the field (Staub and Sozzi, 1984, Staub, 1991, Schwinn and Morton, 1990). The vineyard in Walenstadt provided an excellent possibility to study the population dynamics of *B. fuckeliana* under such conditions (**chapter 1**). In a three year field trial the pathogen population was challenged by only one dicarboximide application in the first year, in the second year no dicarboximides were applied and in the third year one third of the population was challenged by two dicarboximide applications, whereas the remaining two thirds were left untreated. However, one or two applications of dicarboximides in the first and in the third season, respectively, resulted in the increase of the resistant population to a level of 100%. The proportion of re-

sistant strains decreased from harvest in the first year to spring in the second year and remained unchanged during the second year and also during the third year when no dicarboximides were applied. Surprisingly, the population in the vineyard in Walenstadt showed the same behavior as was reported by Leroux and Clerjeau (1985), Martinetti (1986), Pak et al. (1990) and Beever et al. (1991) for populations on susceptible grape varieties that were heavily treated. These results clearly indicate that, in this case, the reduction of the selection pressure achieved by restricted dicarboximide applications due to the use of a less susceptible grape variety could not prevent the buildup of a resistant population in the field.

Martinetti (1986) and Leroux (1991) studied the ability of *B. fuckeliana* to develop cross-resistance against dicarboximide fungicides and the phenylpyrrole fenpiclonil. Their speculations on a common mode of action of dicarboximides and phenylpyrroles were, however, contradictory (see chapter 3). The phenylpyrrole CGA 173506 provided a most suitable tool to study the potential of the fungus to develop resistance and to study the occurrence of cross resistance to the chemically not related dicarboximides. Studies on the variability of sensitivity to CGA 173506 and to vinclozolin, respectively, showed an only small variation in the base-line sensitivity in both active ingredients. CGA 173506 proved to be more active than vinclozolin; by both compounds spore germination was less inhibited than mycelial growth. Dose- response curves were similar in shape. They showed a steep slope in the linear part of the $\int$ -shaped curves. Significance and practical importance of the assessment of the base-line sensitivity is explained in detail in chapter 2. While the variations in sensitivity of wild type strains was small, pretending genetic homogeneity, under the selection pressure of CGA 173506 or vinclozolin, respectively, the genetic flexibility of *B. fuckeliana* became obvious. In comparative studies on the occurrence of laboratory resistance against CGA 173506 or vinclozolin, respectively, the biological phenomenon of the spontaneous emergence of "runaways", that, so far was claimed to be unique in dicarboximide fungicides (Leroux et al., 1977, Schüepp and Küng, 1978), was identically found in the phenylpyrrole.

Most "runaways" that spontaneously occurred on artificial media amended with strongly inhibiting or lethal concentrations of CGA 173506 or vinclozolin, respectively, generally proved to be resistant, however, in some cases they were neither altered in sensitivity to dicarboximides nor to CGA 173506. The assessment of dose-response relationships concerning CGA 173506 and vinclozolin in 34 "runaways" revealed resistance levels from highly sensitive to highly resistant (chapter 3). In contrast to the results obtained from tetrad analysis of (genetically homogenous) sexual progenies (chapter 4) that clearly indicated the existence of 3 distinct resistance patterns for both active ingredients the classification of resistance levels of "runaways" was difficult. Grindle (1979) discussed the hypothesis that the phenotype in a heterokaryon depends on the kinds and proportions of nuclei present. He indicating that mutations in a minority of the nuclear component of a heterokaryon could be masked by the majority of normal alleles in the other components. As in *B. fuckeliana* anastomoses allow the exchange of nuclei, concluding from the results from Grindle (1979) it can be speculated that due to the random combination of sensitive and resistant nuclei theoretically, any resistance level could be reached in asexual progenies. Although Parmeter et al. (1963) in their review on heterokaryosis and variability in plant pathogenic fungi emphasized the need of further evaluation of the concept of heterokaryosis, unfortunately the interplay and the state of activity of resistant and sensitive nuclei in heterokaryons was not studied so far and therefore discussions of possible effects on the resistance level are still mainly hypothetical.

While Leroux et al. (1977) and Schüepp and Küng (1978) found a rapid development of dicarbox-

imide laboratory resistant strains *in vitro*, the selection of resistant strains was much slower under field conditions. First reports about dicarboximide field resistant strains in vineyards were encountered after three years of practical use of these fungicides (Holz, 1979, Schüepp et al., 1982). The occurrence of dicarboximide resistant strains in the field was, however, not correlated with loss of efficacy at that time (Schüepp et al. 1982, Pommer and Lorenz, 1982, Löcher et al., 1985). When the resistance frequencies reached nearly 100% in Swiss vineyards the loss of efficacy of the dicarboximides was also found (Schüepp and Siegfried, 1983). Whereas in the laboratory, growing conditions are optimized and selection pressure is only exerted by the fungicide in the medium, under field conditions the selection pressure is much more complex and a disadvantage in fitness by the acquisition of resistance genes often leads to a retardation of the occurrence of resistant strains (Brent et al., 1990). Comparative studies on the fitness of strains laboratory resistant to vinclozolin and CGA 173506, showed a general decrease. While in **chapter 3** a close correlation of the acquisition of laboratory resistance to both active ingredients and the loss of virulence, osmotolerance and competitive ability was demonstrated, dicarboximide field resistant strains were sensitive to CGA 173506 and showed no loss of fitness. The present studies not only revealed a clear difference in the fitness between field resistant strains and laboratory resistant strains but also in the level of resistance.

Until recently genetics have only been marginally studied in *B. fuckeliana* as the production of apothecia was difficult and few authors reported production of apothecia (Groves and Drayton, 1939, Groves and Loveland, 1953, Lorenz and Eichhorn, 1983, Faretra and Antonacci, 1987, Faretra et al., 1988, Faretra and Pollastro, 1988). As mentioned before (**chapter 4**) Faretra and Pollastro (1991) for the first time provided data on the analysis of the genetic basis of resistance to benzimidazole and dicarboximide fungicides in *B. fuckeliana*. The present studies confirm the results of Faretra and Pollastro (1991) for vinclozolin by using the technique of classical tetrad analysis. In addition (**chapter 4**) laboratory resistance to CGA 173506 was found to be linked with resistance to vinclozolin.

Table 5.1 shows in a combination square all independent combinations of the six alleles (Daf1S, Daf1LR, Daf1HR, Pyr1S, Pyr1LR, Pyr1HR) which are coding for sensitivity, low and high resistance to vinclozolin or to CGA 173506, respectively. From the nine expected phenotypes only four were repeatedly found in the present investigation (shaded squares). Three hypotheses on the genetic basis of the connection between resistance to dicarboximides and to phenylpyrroles were discussed in **chapter 4.**

Genetic studies were extended to two fitness parameters, osmotolerance and pathogenicity, that were described to be altered in dicarboximide (Beever, 1983, Schüepp and Küng, 1978) and CGA 173506 (**chapter 3**) resistant strains (details in **chapter 4**).Vinclozolin/ CGA 173506 double resistant strains showed decreased osmotolerance and reduced pathogenicity on apple when compared to the sensitive strains from the same ascus. In contrast to the laboratory resistant strains, dicarboximide field resistant strains did not show any or only a minute decrease in osmotolerance and pathogenicity on apple. Table 5.1 shows that all strains which are resistant to CGA 173506, independent of the level of resistance, showed an increased osmotic sensitivity too. In contrast dicarboximide field resistant strains (which are sensitive to CGA 173506) and one strain which was reported from Faretra (pers. comm.) to be highly resistant to vinclozolin and sensitive to CGA 173506, were normally tolerant to high osmotic pressure.

Absence of strains highly resistant to dicarboximides in the field and lack of strains resistant to

CGA 173506 but sensitive to vinclozolin, *in vitro* as well as *in vivo*, lead to the following speculations: 1. the acquisition of resistance to CGA 173506 also implies resistance to vinclozolin (and increased osmotic sensitivity) but not *vice versa* and 2. acquisition of high resistance to vinclozolin in the field and resistance to CGA 173506 linked with resistance to vinclozolin either are lethal under field conditions or cause a loss of fitness that has not yet been overcome. A new term is needed to suitably describe this connection of resistance to dicarboximides and phenylpyrroles in *B. fuckeliana* as neither the definitions of cross resistance nor of multiple resistance given by Dekker and Delp, (1985) match in this case.

Table 5.1: Combination square: independent combination of 6 alleles coding for sensitivity, low and high resistance to dicarboximides and phenylpyrroles. Only the phenotypes described in the shaded squares could be stablely maintained in the present investigation.

reaction to vinclozolin / reaction to CGA 173506	*sensitive (=S) EC50 < 1mg/L*	*low resistant (=LR) 1mg/L < EC50 < 30mg/L*	*highly resistant (=HR) EC50 > 30mg/L*
sensitive (=S) EC50 < 0.03mg/L	**S *S*** wild type	**S *LR*** field resistance	**S *HR*** only 1 lab. res. strain (Faretra, pers. comm.)
low resistant (=LR) 0.03mg/L < EC50 < 0.3mg/L	**LR *S*** not found	**LR *LR*** only few strains ● lab-resistance	**LR *HR*** not found or unstable ●
highly resistant (=HR) EC50 > 30mg/L	**HR *S*** not found	**HR *LR*** not found	**HR *HR*** frequent ● lab-resistance

● hypersensitive to increased osmotic pressure measured as glucose tolerance

Summary

In the present investigations genetic variability in *B. fuckeliana* was studied with regard to fungicide resistance to vinclozolin and the phenylpyrrole CGA 173506.

Population dynamics of *B. fuckeliana* resistant to dicarboximides were studied in a vineyard at Walenstadt, Switzerland on the grape variety Blauburgunder, clone Mariafelder, which is weakly susceptible to grey mould and therefore dicarboximides were rarely used. In a three year field trial, one and two applications of dicarboximides in the first and in the third season, respectively, resulted in the increase of the resistant population to a level of 100%. The proportion of resistant strains decreased from harvest in the first year to at most 20% in the spring of the second year and further decreased but did not disappear during the second year nor during the third year when no dicarboximides were applied. Restricted applications of dicarboximides did not prevent the buildup of a resistant population. This population behaved like populations that were selected under a heavy selection pressure.

Variability in the sensitivity to CGA 173506 and vinclozolin of *B. fuckeliana* isolated from strawberries from fields that never ever received any fungicidal treatments was investigated. Spore germination was less inhibited than mycelial growth by both active ingredients; EC50 values ranged from 1.7- 2.0mg vinclozolin/L and 0.072-0.19mg CGA 173506/L for spore germination and from 0.12-0.27mg vinclozolin/L and 0.0027-0.016mg CGA 173506 for mycelial growth.

Resistance to CGA 173506 and to vinclozolin was genetically fixed that is, it was not caused by physiological adaptation. "Runaways" (strains spontaneously arising on fungicide amended agar) generally proved to be resistant to vinclozolin and to CGA 173506, however some of them were sensitive. Strains with a reduced sensitivity to CGA 173506 but not to vinclozolin were never found. Acquired laboratory resistance was generally stable after several transfers on apples and on pea agar, respectively, however, some strains lost their resistance. Investigations on the morphological appearance of "runaways" and the level of resistance revealed a correlation of morpho-type and resistance level which was however not clear cut in all cases. Conidial dimensions and number of nuclei of vinclozolin and CGA 173506 laboratory resistant strains did not differ. However, differences in fitness were significant: Acquisition of laboratory resistance to CGA 173506 and vinclozolin was correlated with loss of virulence, osmotolerance and competitive ability. Dicarboximide field resistant strains were as virulent as sensitive strains and their osmotolerance was not altered. Virulence and osmotolerance, however, decreased when they additionally acquired laboratory resistance to vinclozolin and CGA 173506.

Homokaryotic sexual progenies of heterokaryotic sensitive strains or heterokaryotic strains having acquired resistance in the field to dicarboximides, or in the laboratory to dicarboximides and CGA 173506, respectively, were produced using field isolates from naturally infected strawberries and grapes. Resistance to CGA 173506 was inherited by one major gene which was termed as gene Pyr1. Tetrad analysis of sexual progenies confirmed the results from Faretra and Pollastro (1991) who found, by random spore analysis, resistance to vinclozolin to be inherited by three alleles (Daf1S, Daf1LR and Daf1HR) of the major gene Daf1. Based on analogy between the results in dicarboximides and phenylpyrroles, it is suggested that resistance to CGA 173506 is inherited by three alleles (Pyr1S (sensitive), Pyr1LR (low resistant) and Pyr1HR (highly resistant)) of the major gene Pyr1. In tetrad analyses Pyr1 and Daf1 did not segregate independently. Dicarboximide field resistant strains were sensitive against CGA

173506, whereas laboratory resistant strains showed double resistance against vinclozolin and CGA 173506. Three hypotheses considering close linkage of Pyr1 and Daf1 or identity of the two genes are discussed.

Osmotolerance and pathogenicity of sexually produced (homokaryotic) strains was assessed. Strains which were resistant to dicarboximides and to CGA 173506 were less virulent and showed a decreased osmotolerance, irrespective of the level of resistance. Dicarboximide field resistant strains, however, while as virulent as sensitive strains, did not show any or only minute differences in osmotolerance when compared to the sensitive strains from the same ascus.

Zusammenfassung

In der vorliegenden Arbeit wurde die genetische Variabilität von *B. fuckeliana* im Zusammenhang mit der Enstehung von Fungizidresistenz gegenüber Vinclozolin und dem Phenylpyrrol CGA 173506 untersucht.

Populationsdynamische Vorgänge in einer Dicarboximid-resistenten Population wurden in einem dafür besonders geeigneten Rebberg in Walenstadt, Schweiz, untersucht. Die dort angebaute Traubensorte Blauburgunder, Klon Mariafelder, weist gegenüber der Graufäule eine reduzierte Anfälligkeit auf, weshalb Dicarboximidfungizide nur wenige Male eingesetzt wurden. In einem dreijährigen Feldversuch führten ein bis zwei Dicarboximidspritzungen im ersten und im dritten Versuchsjahr zu einem Anstieg der resistenten Population bis auf 100%. Der Prozentsatz resistenter Stämme nahm von der Ernte des ersten Versuchsjahrs bis zum Frühjahr des zweiten Versuchsjahrs bis auf maximal 20% ab. Im Verlauf des zweiten und dritten Versuchsjahres ging er weiter zurück, sofern keine Dicarboximidbehandlungen vorgenommen wurden. Resistente Stämme wurden jedoch nicht vollständig verdrängt. Eine reduzierte Anzahl Dicarboximidbehandlungen verhinderte den Aufbau einer resistenten Population nicht. Diese Population verhielt sich hinsichtlich populationsdynamischer Vorgänge gleich wie resistente Populationen, die unter hohem Selektionsdruck entstanden sind.

Die natürliche Variabilität in der Sensitivität gegenüber CGA 173506 und Vinclozolin von *B. fuckeliana*- Erdbeerisolaten aus nie mit Fungiziden behandelten Parzellen wurde untersucht. Beide Fungizide hemmten die Sporenkeimung weniger als das Myzelwachstum, wobei CGA 173506 die höhere Wirkung zeigte als Vinclozolin. EC50 Werte für Sporen-keimung lagen zwischen 1.7- 2.0mg Vinclozolin/L und 0.072-0.19mg CGA 173506/l, für Hemmung des Myzels zwischen 0.12-0.27mg Vinclozolin/l und 0.0027-0.016mg CGA 173506/l.

Sowohl die Resistenz gegen Vinclozolin wie auch gegen CGA 173506 sind konstitutionell (genetisch fixiert). "Ausreisser" (auf Fungizidmedium spontan wachsende Isolate) waren resistent gegenüber CGA 173506 und Vinclozolin; es konnten aber auch Stämme gefunden werden, deren Sensitivität gegenüber diesen Wirkstoffen nicht vermindert war. Stämme, die eine reduzierte Sensitivität gegenüber CGA 173506, nicht aber gegenüber Vinclozolin aufwiesen, wurden nicht gefunden. Laborresistenz gegenüber CGA 173506 und Vinclozolin war im allgemeinen stabil, obwohl einige Stämme ihre Resistenz nach mehrmaliger Passage über Äpfel oder über Erbsenagar verloren. In der Mehrzahl der untersuchten Fälle bestand ein Zusammenhang zwischen Erscheinungsform von "Ausreissern" und deren Resistenzniveau. Bezüglich Längen- und Breitenmessungen sowie Kernzahl konnten laborresistente Stämme nicht von sensiblen unterschieden werden. Erworbene Laborresistenz war jedoch mit einem Verlust an Virulenz, Osmotoleranz und Konkurrenzfähigkeit korreliert. Dicarboximid-feldresistente Stämme zeigten keinen Unterschied in ihrer Virulenz und ihrer Osmotoleranz im Vergleich zu sensiblen Stämmen. Beide Eigenschaften waren jedoch stark vermindert, wenn feldresistente Stämme zusätzlich Laborresistenz gegenüber CGA 173506 und Vinclozolin erworben hatten.

Homokaryotische Stämme wurden aus Kreuzungen von heterokaryotischen sensiblen mit heterokaryotischen Dicarboximid-feldresistenten oder Dicarboximid- und CGA 173506- laborresistenten Stämmen gewonnen. Die heterokaryotischen Freilandisolate stammten von Erdbeeren und Trauben. Nach einer Ruheperiode bei erniedrigter Temperatur wurden reife Sklerotien einzelner Stämme mit einer Suspension von Mikrokonidien, Makrokonidien und Myzelfragmenten verschiedener Stämme sper-

matisiert. Resultate aus Kreuzungsexperimenten zeigten, dass die Laborresistenz gegen CGA 173506 durch ein Strukturgen vererbt wird, welches als Pyr1 bezeichnet wurde. Die Tetradenanalyse bestätigte die Resultate von Faretra und Pollastro (1991), die aufgrund von Massensporenanalysen schlossen, dass die Resistenz gegen Dicarboximidfungizide durch das Hauptgen Daf1, welches in 3 Allelen (Daf1S, Daf1LR und Daf1HR) vorkommt, vererbt wird. In Analogie zu den Resultaten, die bei der Untersuchtung der Vererbung der Dicarboximidresistenz gefunden wurden, wird angenommen, dass das Phenylpyrrolresistenzgen ebenfalls in drei verschiedene Allelen (Pyr1S (sensibel), Pyr1LR (mittelresistent) and Pyr1HR (hochresistent)) vorkommt. In Tetradenanalysen konnte keine unabhängige Segregation zwischen Pyr1 und Daf1 nachgewiesen werden. Dicarboximid- feldresistente Stämme zeigen keine verminderte Sensibilität gegenüber CGA 173506. Laborresistente Stämme sind dagegen in allen Fällen sowohl gegen CGA 173506 als auch gegen Vinclozolin resistent. Drei Modelle welche ein enge Kopplung der Gene Pyr1 und Daf1 oder die Identität der beiden Gene berücksichtigen werden diskutiert.

Osmotoleranz und Pathogenität der sexuellen (homokaryotischen) Nachkommen wurde untersucht. Stämme die eine Laborresistenz gegenüber Dicarboximiden und CGA 173506 aufwiesen, zeigten sowohl eine verminderte Virulenz wie auch eine reduzierte Osmotoleranz. Sexuell erzeugte Nachkommen Dicarboximid-feldresistenter Stämme zeigten jedoch weder in der Virulenz noch in der Osmotoleranz eine deutliche Veränderung im Vergleich mit sensiblen Stämmen aus dem gleichen Ascus.

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