TCP-SLC-3402

MANUAL ON FUNGICIDES AND FUNGICIDE RESISTANCE MONITORING IN BANANA

Luis Pérez-Vicente

Senior Plant Pathologist, INISAV, Ministry of Agriculture, Cuba.

Prepared for a Regional Workshop on Fungicides and Fungicide Resistance in Banana 17-22 June 2013, Roseau, Dominica

TCP-SLC-3402 Project - *Development of Integrated Programmes and Action Plans for Black Sigatoka Disease Management in five countries of the Caribbean*

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

June 2013

TCP/SLC/3402

MANUAL ON FUNGICIDES AND FUNGICIDE RESISTANCE MONITORING IN BANANA

Luis Pérez-Vicente

Senior Plant Pathologist, INISAV, Ministry of Agriculture, Cuba.

Prepared for a Regional Workshop on Fungicides and Fungicide Resistance in Banana. 17-22 June 2013, Dominica

TCP-SLC-3402 Project - *Development of Integrated Programmes and Action Plans for Black Sigatoka Disease Management in five countries of the Caribbean*

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

June 2013

Table of Contents Page

1. INTRODUCTION

1.1. Banana / Plantain crops and their pathogens

Banana and plantain (*Musa* spp.) are grown throughout the tropical and subtropical regions of the world. They are a key staple food in many developing countries and a source of income for subsistence farmers.

Bananas are one of the main agricultural crops in the world, and the main fruit crop, with an annual production of about 117 Mt (Lescot, 2011). Bananas are also a major, multi-billion dollar export commodity primarily for consumption in developed countries. International trade of bananas represents 14 Mt/year with a value of more than US$ 4 billion (Loeillet, 2005). The production of bananas for this international trade is in a small number of tropical countries mainly located in Latin America (80%), and in African and Asian countries. In all these countries, the banana industry is economically important and a source of direct and indirect employment. But one of the main problems is that it relies on a narrow genetic base, the Cavendish subgroup, which exposes the industry to threats from a number of key pests and diseases.

Globally, plantains are grown on 5.4 million ha, with a production of 36 million Mt.

Apart from their importance to the national economies, both plantain and banana play an important role in food security and livelihood of growers.

Banana and plantain are attacked by different pathogens that affect plant development, cause yield losses and reduce fruit quality. The most important are Sigatoka leaf spots caused by *Mycosphaerella musicola* and *Mycosphaerella fijiensis* and crown rot caused by a fungal complex composed by *Colletotrichum musae*, *Fusarium pallidoroseum*, *Verticillium theobromae*, *Lasiodiplodia theobromae and Fusarium spp.*, among others (Stover, 1972; Pérez *et al.* 1996). From an economic point of view, banana and plantain leaf spots caused by *Mycosphaerella fijiensis* Morelet (black Sigatoka/black leaf streak) and by *M. musicola* Leach ex Mulder(yellow Sigatoka), can be considered, the two most serious diseases of *Musa* spp.

Over the past 50 years, a large number of new active ingredients have been discovered for control of fungal diseases and there is a higher level of knowledge and understanding of their structure and action mechanisms. These active ingredients pose lower toxicological risk to producer, consumers and environment due to more specific sites and mechanisms of action. A better knowledge of the properties of naturally-occurring compounds with fungicidal properties resulted in the development of new fungicides with excellent fungicidal and toxicological properties suited for use in current modern agriculture. These active ingredients used in the framework of integrated management programs are formidable tools (and frequently the only ones available) to prevent losses caused by diseases and improve yields in crops. The higher selectivity and more specific modes of action provide the opportunity for a selection of pathogen populations that have reduced sensitivity or resistance to the pesticides.

Black Sigatoka leaf spot management requires frequent application of systemic fungicides. *Mycosphaerella fijiensis* has a significant capacity of variation and adaptation. The *Mycosphaerella* leaf spots-Musa spp. pathosystems are continuous in time and space and the pressure of selection of populations with low sensitivity and resistance to the fungicides in use is high.

The frequent use of monosite active ingredients in the BSD control can result in the selection and build up of fungal populations tolerant to these products, with decline in control levels and a concurrent increase of protection costs. For this, it is of paramount importance that the

management program includes a systematic monitoring of the sensitiveness of fungal populations to the main active ingredients being used for BSD control.

In the present manual the main fungicide groups used in banana for disease management, their mechanism of action and resistance as well as theory of the process of selection of resistant population are reviewed. The current recommended protocols developed for testing the sensitiveness of populations to the main fungicide groups are included.

The purpose of the Workshop is to create the necessary capacity on the countries to systematically carry out monitoring tests in order to prevent losses from the emergence of resistant populations.

1.2. Black Sigatoka Disease

1.2.1. History and symptoms

The fungal pathogen *Mycosphaerella fijiensis* (anamorph *Pseudocercospora fijiensis*) causes black Sigatoka disease (BSD) on the majority of edible banana cultivars grown worldwide. The disease appeared in the Americas (in Honduras) in 1972 and disseminated in the Caribbean in two independent introductions: In 1990 in Cuba (then Jamaica, Dominican Republic, Haiti and Puerto Rico) and in 2003 Trinidad and Tobago, spreading along the main commercial banana-producing countries of the Lesser Antilles (Grenada, Dominica, St. Lucia, St. Vincent and the Grenadines, Martinique, Guadeloupe) as well as Guyana in mainland South America. The status of BSD in the other countries of the Caribbean is currently not known.

The disease cycle of *M. fijiensis* consists of four distinct stages that include spore germination, penetration of the host, symptom development and spore production. After a period of epiphyllic growth of generally 2-3 days, germ tubes penetrate the stomata. Under favourable conditions, the first symptoms appear generally 10-14 days after incubation (Figure 1); the symptoms then gradually evolve from stage 1 (streaks) to first stage of spots (necrosis, transition period) and stages 5 and 6 (Fouré, 1982). Conidia are produced in early phases (stages 2-4); short-distance dispersal is primarily by water, although wind-borne dispersal may also occur. Ascospores, produced at later stages, are wind-dispersed (after the pseudothecia burst) and are dispersed at longer distances than conidia.

Six stages of lesion development following infection by the fungus have been described (Fouré, et al., 1984; Figure 1):

First stage: is a faint reddish-brown speck less than 0.25 mm diameter visible on the underside of the leaves.

Second stage: is characterized by an elongation of the specks turning into reddish brown streaks along the long axis of the streak, parallel to the leaf veins.

Third stage: the streaks coalesce, reaching about 20 x 2 mm, and change to dark brown. Conidia are produced at the second and third stages. If the streaks are numerous at this stage, the entire leaf can turn black.

Fourth stage or the first spot stage: when lesions develop into spots becoming fusiform or elliptical with water soaked borders.

Fifth stage: Is reached when the dark brown or black centre of the spots become depressed and the spots are surrounded by a yellow halo. At this stage conidia as well as ascospores are produced.

Sixth stage: the centre of the spot is light gray and dry. Where the spots coalesce, entire sections of leaves become necrotic. In these sections, ascospore production is high. After flowering and fruit production, plants can lose all leaves (Figure 2).

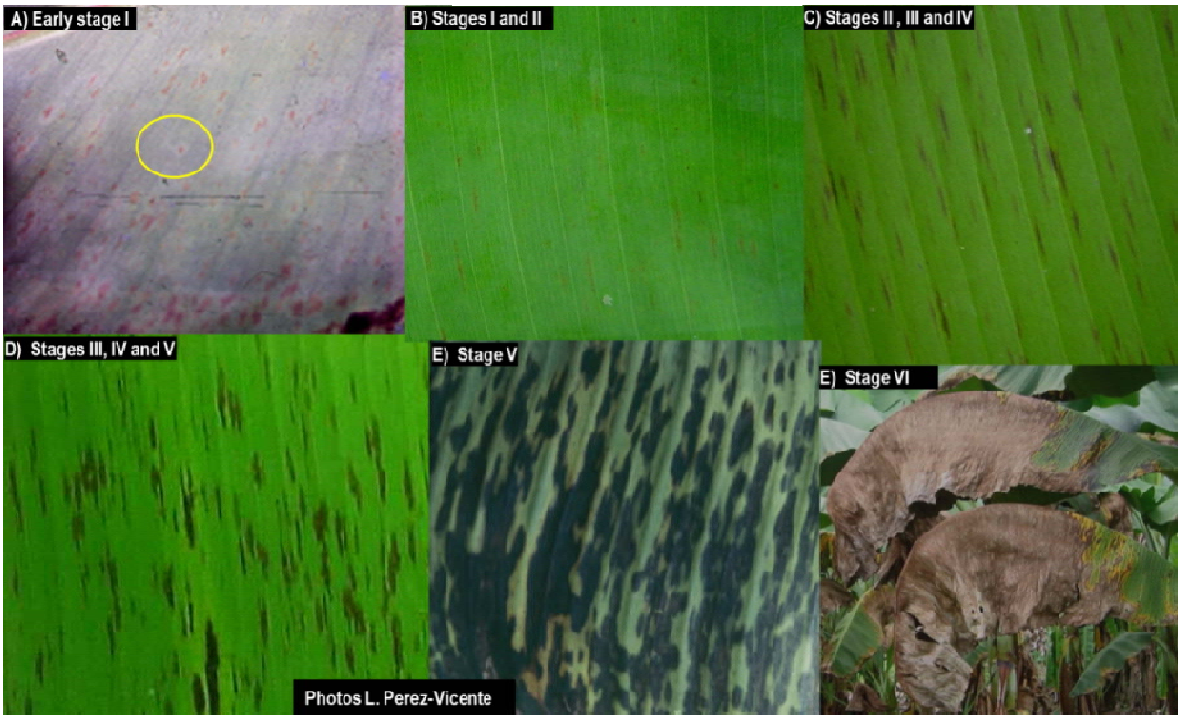


Figure 1. Black Sigatoka symptoms stages.

1.2.2. The pathogen

Mycospherella fijiensis belongs to the class Dothideomycetes, order Capnodiales and family Mycosphaerellaceae and is the most destructive member of the 'Sigatoka disease complex', which includes *M. musicola* (anamorph *Pseudocercospora musae*), and *M. eumusae* (anamorph *P. eumusae*), which causes Eumusae leaf spot disease. The three species are hemibiotrophic and heterothallic (Stover, 1963; Mourichon and Zapater, 1990).

The fungus produces multi-cellular conidia from conidiophores in culture and *in planta*, in the latter case arising from stomata primarily on the abaxial (lower) surface of the infected leaves. Conidiophores arise from hyphae present in the sub-stomatic chamber and can produce multiple conidia from a single conidiophore (Figure 2). Conidiophores are produced on the lower surface of initial specks (Stage 1, Figure 1A) or at the first streak stage (Stage 2, Figure 1B) up to the second spot stage (Stage 5; Figure 1 E). Conidia are produced almost continuously between the second streak (Stage 3; Figure 2B) and second spot (Stage 5; Figure 1D)

In BSD spot samples from Cuba (Pérez-Vicente, 1993; Pérez-Vicente, 1996), **conidiophores** were observed to develop from four to six engrossed cells of the substomatic chamber and emerge through stomata, in fascicles of two to four. The conidiophores are pale brown, straight to variably curved, with a wider basal cell and 0-5 septa, 27-71 x 3-5 µm and with up to six scars at the apex or on a light lateral shoulder. At the 8- to 12-day stage, single-spore isolations cultures developed hyaline conidiophores producing conidia at the extreme (Figure 4). **Conidia** are obclavate, hyaline to brown olive in colour, 5-8 septa, 27-110 x 2-5 µm and with a well marked hilum that allows easy dispersal by wind. **Pseudothecia,** which appear in spots at stages 4 to 6, are amphigenous and erumpent, with a papillated dark ostiole, and walls composed of two or

three layers of polygonal dark brown cells. They are more abundant on adaxial side of leaves, with a diameter between 43 and 86.5 µm, bitunicated hyaline ascii, without paraphyses, with two rows of hyaline, fusiform to cleaved bi-celled **ascospores,** having a larger anterior cell and a marked constriction at the level of septa, 12-16 x 2.5-5.0 µm. Spermogonia are more abundant in the adaxial side of leaves, globose, obpyriform, with pale brown walls of 23-55 µm of diameter with an ostiole slightly prominent that emerges by stomata and have rows of unicellular, bacilliform, hyaline trunked by its extremes spermatia of 2.0-4.5 x1.5-3.0 µm.

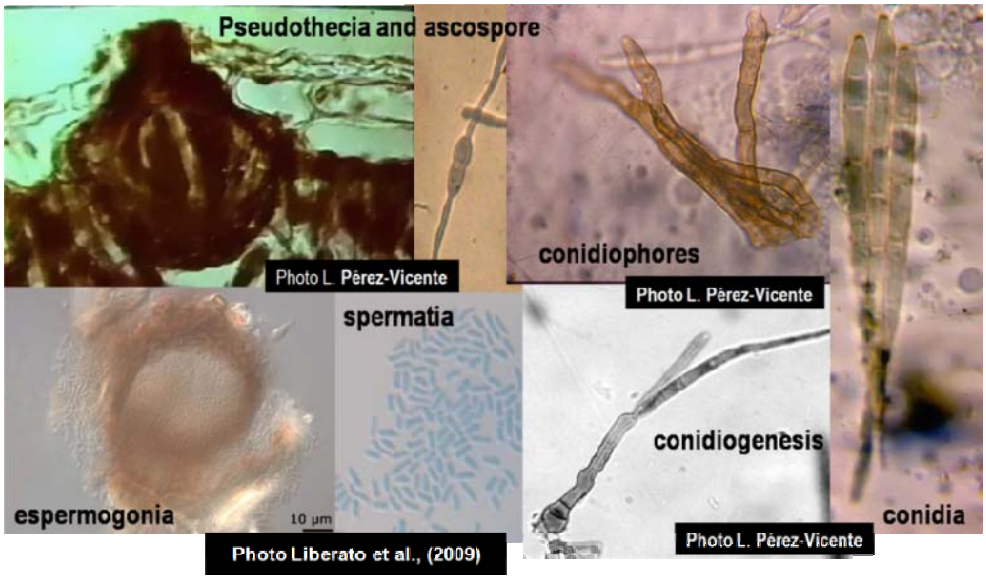
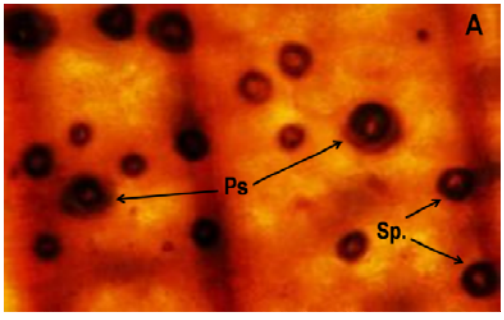


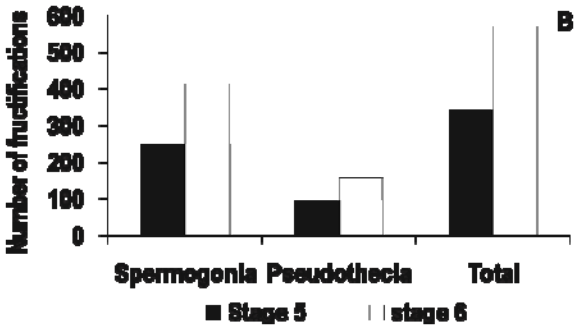
Figure 2. *M. fijiensis* morphologic traits.

Both anamorphs and teleomorphs of *M. fijiensis* can be concurrently present on infected leaves. Pseudothecia and spermogonia are produced in stage 5 and 6 and are more abundant on adaxial (upper) than abaxial (lower) surface of spots. (Figures 3 a-c)

Abundant development of asexual and sexual spores takes place in warm, rainy weather. Wet leaf surfaces favour spermatia release. This is followed by fertilization of protopseudothecia and development of ascospores, which are released by rainfall and in the presence of a water film on the leaves. Duration of leaf wetness is important for the completion of development and release of pseudothecia and ascospores (Figure 4). Ascospore release is important in the first 30 minutes after the leaves are wetted with spots and is completed in a period of an hour.

After release, ascospores very readily germinate in a saturated atmosphere or in the presence of a water film on leaves (Figure 6).





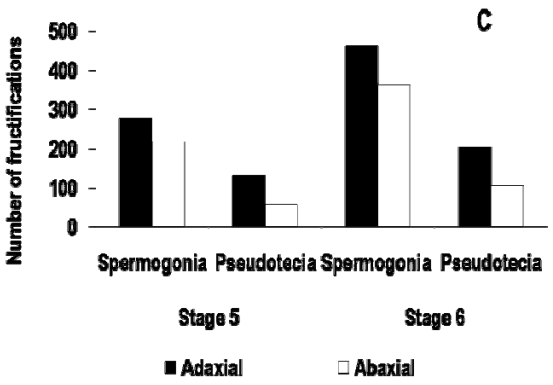


Figure 3. Pseudothecia and spermogonia production on spots

a) **Pseudothecia (Ps.) and spermogonia (Sp.) on BSD spots.**

b) **Pseudothecia and spermogonia in spots at stage 5th and 6th. (Montero and Pérez- Vicente, unpublished).**

c) **Number of pseudothecia production in abaxial and adaxial surface of BSD spots.**

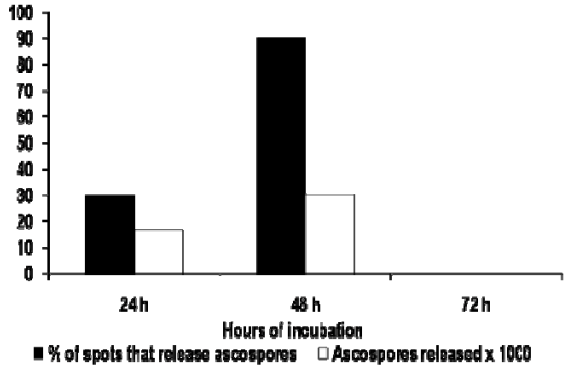


Figure 4. Influence of the period of incubation at high relative humidity of leaves fragments with BSD spots, on frequency and intensity of *M. fijiensis* ascospores release (Pérez, 1998).

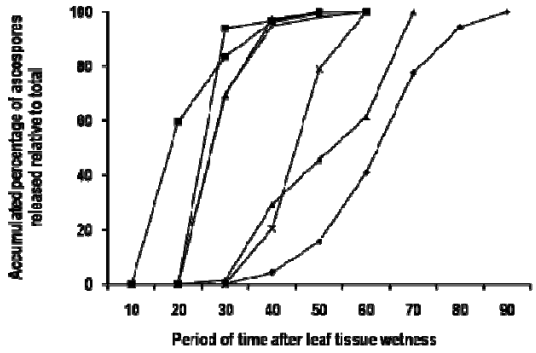


Figure 5. Dynamics of *M. fijiensis* ascospore release after leaf wetting. (Data of seven replications; Pérez, 1998)

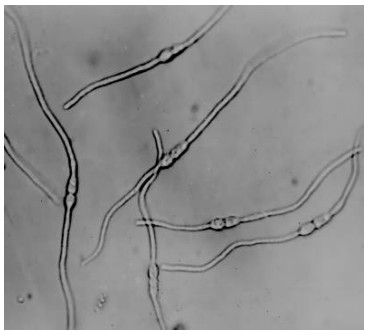


Figure 6. *M. fijiensis* ascospores germinated on water agar in Petri plates

1.3. Other Mycosphaerella species associated with Sigatoka leaf spots

Different *Mycosphaerella* species are frequently associated to banana leaf spots and necrotic tissues of banana (Stover, 1963; Stover, 1969; Pérez, 1980; Pérez, 2002). Among them, are *Mycosphaerella minima* Stahel (Stahel, 1937) and *Mycosphaerella musae* Spegazzini.

Mycosphaerella musae is commonly found associated with black Sigatoka disease and other leaf spots on banana leaves (Stover,1969; Pérez,1980; Pérez, 2003). It causes leaf speckle disease in Australia and South Africa, where it produces epidemics that require fungicide treatments for control. Pseudothecia of these species are similar to those of *M. fijiensis and M. musicola* but ascospores are shorter and narrower (Figure 6). Stover (1977; 1994), reported the association between a non-virulent *Cercospora* with verrucose walls developed in simple conidiophores in banana leaves and *M. musae*.

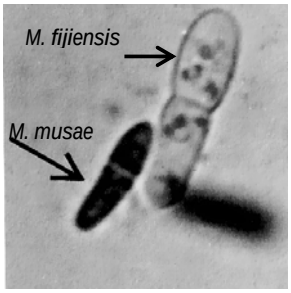


Figure 7. *M. fijiensis* (12-16 µm) and *M. musae* ascospores (<13 µm)

Mycosphaerella minima Stahel. can be frequently found as saprophyte in Sigatoka spots (Stover, 1969; Pérez, 1980; Pérez, 1993; Figure 8); the pseudothecia are 25 - 37 µm in diameter (average 31µm) with one or two ascii; ascospores are 20 – 25 µm, with a marked constriction at the level of the central septum, a more acute apical cell than the rest of *Mycosphaerella* species and one or two easily visible oil drops. This species can also be differentiated from *M. fijiensis* and *M. musicola* ascospores by its sinuous and thinner fast-growing germ tubes. Based on these characteristics, *M. minima* ascospores are easily and quickly distinguished from those of *M. musicola* and *M. fijiensis* (Figure 8).

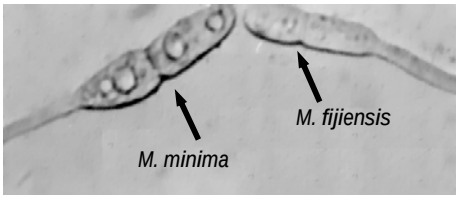


Figure 8. *M. fijiensis* (12-16 µm) and *M. minima* ascospores > 18 µm)

2. FUNGICIDES - TERMINOLOGY AND CONCEPTS

Many modern compounds are not really "*ides*". The word fungicide suggests that the compound kill the fungus in a way more or less without selectivity, but it is no longer the case. Most of fungicides act fungistatically at the beginning and fungicide action is obtained after a prolonged interaction with the fungus. Even the ***"multisite inhibitors***" on occasions present selectivity in spite of their unspecific mechanisms of action. A more useful term is ***xenobiotic*** that describe a molecule that is a stranger to the plant. Many fungicides, insecticides and herbicides are therefore xenobiotics.

The term ***systemic fungicide*** is applied to fungicides that are systemic or move within the plants. As many chemical compounds are converted inside the plant, the chemical molecule that is toxic to the pathogen is called ***toxophore***.

"Multisite" is a compound that acts in different sites of fungal metabolism. ***Unisite*** on the contrary, are compounds that block processes at very specific points of metabolism, frequently regulated by a single pair of genes.

2.1. Transport systems in plants

The presence of a cell wall prevents direct contact between plasma membranes of adjacent cells: consequently the plant body is divided in two compartments (Münch, 1930; Esau, 1965): the **symplast** and the **apoplast**.

The xenobiotics can enter into the plant and move in direction of evapotranspiration stream or with photosynthates to roots or in both directions. These patterns of movement are regarded as:

Apoplastic; is referred to transport to long distances in the coherent network of free space, cell walls, cuticle and non-living cells as xylem vessels and trachea (= apoplast). It can be: a) ***euapoplastic*** if only the xylem elements or apoplast are involved; *b)* ***pseudoapoplastic*** if both apoplasts are involved and they occasionally pass through or are retained by cell protoplasts. This implies that xenobiotics are accumulated based on their solubility at the points of higher stomatal density where transpiration is more intense. It also implies upward movement from the roots to stems, petioles and leaves and from the base and midrib to the border of leaf lamina but not in the reverse direction.

Symplastic*;* is related to long-distance movement in the coherent network of living protoplasts connected by plasmodesmata, wherein the xenobiotic ionize and move in the phloem with the pathway of the photosynthates to the roots. The symplast is the living part of the plant covered by membranes (protoplasts and plasmadesmata including the living cells companying the xylem vessels).

Ambimobil. Xenobiotics can move in both directions in the symplast and apoplast (Edginton, 1981; Neumann and Jacob, 1995).

Locosystemic**:** xenobiotics cannot move long distances from application site, being located in the applied organ of the plant.

2.1.1. Physiological requirements for systemic movement of fungicides

Membrane permeability

Most xenobiotics move mainly upward and are considered apoplastics. However, all systemics are capable of going through plasmalemma membrane and enter the protoplasts. When they enter through the roots, due to the Casparian band barrier, xenobiotics have to pass through protoplast to endoderm and probably enter through vacuoles and other membranes that envelope the organelles such as mitochondria and chloroplasts. (Edginton, 1981; Neumann and Jacob, 1995).

Selective toxicity

Modern systemic fungicides have necessarily to be selective to prevent phytotoxic effects. As a systemic xenobiotic enters the membranes, it should not be toxic to the host cells of plants,

but act against the objective fungal pathogen. Obvious exceptions are ingredients that alter plant physiology and are not directly toxic to the pathogen. Toxophores must be very selective in terms of chemical structure and site of action. E.g. carbendazim (primary toxophore of benomyl) has specific affinity by the ß protein of microtubules in the fungal cell but not of the plant. Carboxin is selectively toxic to the succinil dehydrogenase complex of the fungus, but not that of the plant. Metalaxyl and its active isomer mefenoxam selectively block a specific polymerase A preventing RNAase synthesis in oomycetes but not in plant and fungi.

This ***unisite*** specificity unfortunately also results in toxic selectivity among different taxonomically distant fungal groups. Their spectrum of activity with regard of multisite compounds is narrower and inclusive in particular taxa.

Bases of selectivity are due to (Lyr, 1985):

- Differences in accumulation of products in cells.
- Different structures of receptors in the object systems
- Different capacities to toxify (activation) the active ingredient
- Different capacities to detoxify the active ingredient.
- Different degrees of importance of the receptor on the survival system of the fungus

Metabolic stability

Since systemics have to enter into the symplast, they are subjected to the metabolism of fungal cell. As such, a high degree of metabolic stability is necessary for the systemic control of the pathogen.

Cuticle penetration

Systemic fungicides are primarily used as foliar spray treatments. Also important are applications to seeds, especially in cereals and grains, to soil in greenhouses, and as paints and injections to tree trunks. The cuticle is the most important barrier. The natural role of cuticle is to prevent leaf desiccation. Hence, the water that is the medium used in sprays, remains on the surface and eventually evaporates. Fungicides need to penetrate cuticle, diffuse through it and penetrate the apoplast under the spray drops. They are formulated as emulsifable or oil dispersible concentrates to allow the fast liquid:liquid fractioning in water and then in cuticle. If a xenobiotic is very lipophilic, penetration is fast but then it can be long retained in the cuticle waxes, delaying the translaminar diffusion and apoplast release (Edginton, 1981). Very hydrophilic compounds can be more slowly diffused in cuticle but can be distributed from cuticle to apoplast over a longer period of time, resulting in a higher absorption. The hydrophilic- lipophilic balance is also important in fungi-toxicity, membrane penetration and translocation. Cuticle penetration is usually obtained by the formulations or by adsorption of a labile chemical group that can release the toxophore after leaf penetration (e.g. benomyl and methyl thiophanate). Non-polar substances such as bases and acids in non-ionic forms better penetrate the lipoproteic base of plasmalemma. Xenobiotic compounds are more easily taken up by germ tubes than by spores (Hassall, 1982).

Solubility in water

Water solubility of xenobiotics do not control permeability of membrane *per se*; it is however important to release the xenobiotic on the surface of the plant in a soluble state. In seed and soil treatment, the xenobiotic solubility is important. The amount of xenobiotic available in the

initial water that seed absorbs is important in efficacy; from this reason, carbendazim and methyl thiophanate are taken up better than benomyl in seed treatments.

2.2. Chemical requisites for systemics

There are hydrophilic-lipophilic requisites that govern absorption and movement of xenobiotics in plants. There are also very different requisites for transport in plant phloem.

2.2.1. Xylem translocation

Xylem translocation takes place in trachae and vessels that form a continuous network of elongated dead cells with more or less lignified secondary walls. The pattern and range of movement will depend of the potential gradient between the air and the soil. Then the transport is usually directed from the roots to the transpiring organs, usually the leaves. Factors that control the intensity of transpiration such as relative humidity, temperature, light, phytohormones, (especially ABA) can affect the rate of translocation and distribution of xenobiotics dissolved in xylem sap (Neumann y Jacob, 1975). The distribution has the following peculiarities:

- Substances accumulates in high transpiration sites (extremes and borders of leaves)
- Transports and organs with low transpiration (fruits and young leaves) are very limited.
- Substances that move in xylem have no downward movement in the expanded leaves. If applied in the base of the leaves, they move to the tips and extremes.

Xenobiotics are absorbed by roots along with water, mainly by the hair zone located between 1-50 mm from the extreme of roots. Rhizoderm, in contrast with leaves, is not covered by a well-developed cuticle and does not constitute a barrier for xenobiotic absorption from soil (Esau, 1975). In roots, absorption is a passive process thats depend on active ingredient concentration in soil, the rate of respiration of the plant (Edgington, 1981), as well as the adsorption capacity of organic matter in soil that is related to log P octanol - water partition coefficient. A high log P value indicates that the substance will bio-accumulate and that the concentration in soil solution will reduce. Once a xenobiotic enters the roots, it will move if log P is between -0.5 and 3.5. Apparently, the plant progressively immobilizes xenobiotics if log P is > 2 and no release to xylem occurs if log P is 4. The xenobiotics with a high log P are immobilized rapidly in roots, in Casparian band or in the lipids of the symplast. The partition is a passive retention and as more water goes through roots, more xenobiotics are released to the transpiratory stream. So, total absorption is function of log P values and time. The xenobiotic that have a low log P value are not systemic due to the impossibility of passing through the plasma membrane.

Absorption of xenobiotics by roots can be characterized by a concentration factor in roots (CFR):

CFR = **xenobiotic concentration in the root/ concentration of xenobiotic in external solution**.

Xenobiotics that enter transpiration stream are not limited to xylem vessels but also laterally move to other tissues.

In practice, systemic fungicides to which responses are expected in leaves, should have log P values in the range of 0.5 and 3.0. The more lipophilic compounds (log P between 3 and 5) act more favorably in roots, as they stay confined on them. In general terms, hydrophilic compounds are transferred through soil and are readily absorbed by root, while lipophilic ones have a retarded movement in soil and a long process of absorption by the root.

Figure 9 provides the log P of different fungicides used in banana relative to their mobility.

Active ingredient	Log P
benomyl	1.4
methyl thiophanate	1.5
spiroketalamine(1)	2.8
pyrimethanil (1)	2.8
azoxystrobin	2.5
cyproconazole	2.9
triadimenol	3.3
epoxiconazole	3.4
tebuconazole	3.7
propiconazole	3.7
flusilazole	3.7
hexaconazole	3.9
bitertanol	4.1
difenoconazole	4.3
trifloxystrobin	4.5

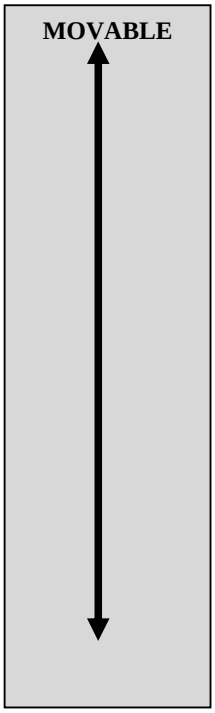


Figure 9. Octanol : water coefficient (Log P) of different fungicides relative to their mobility in the plant.

(1) Although they have low log P values, transportation in the leaves is very low.

2.2.2. Transport in the phloem

In the phloem, transportation occurs in a continuous network of sieve tubes consisting of long living cells, with perforations in the cell wall named sieve areas and sieve plates. The nuclei and the tonoplast disappear in fully differentiated sieve elements. The pH of sieve elements protoplast is 7.2 - 7.8, characteristically high with regard to the parenchymatic cells, due to the activity of the ATPase enzymes that pump protons inside the apoplast.

Sieve elements and companion cells are an anatomical and physiological unit. Both cell types appear to be involved in the accumulation of assimilates; meanwhile longitudinal movement in expanded green leaves and storage organs occurs exclusively in the cribs tubes.

Ambimobile xenobiotic movement in phloem has the following peculiarities:

- Distribution of xenobiotics in phloem is unavoidably connected with the transport of photo-assimilates produced in the photosynthesis. There is no export of xenobiotics in very young developing leaves. When they reach two thirds of their total development, the leaves become a source that exports xenobiotic together with the products produced.
- When the transportation of assimilates stops, the movement of xenobiotics also stops. Xenobiotics that interfere with the process of sucrose synthesis via the process of accumulation or that damage the membranes, stop the phloem translocation, and hence, their own transport. This process is known as auto limitation of mobility by phytotoxicity (Chaleef and Mauvais, 1984).

Following application to the roots, only a small proportion of mobile xenobiotics are transported to the upper parts, while, ambimobile shows systemic behavior of xenobiotics in xylem. In contrast with the systemic xenobiotics in xylem that do not exhibit particular structural

characteristics, the ones that are mobile in phloem are usually acids (in many cases dependent of carboxylic, phenolic, hydroxylic groups). It has been shown that the loss of the COOH groups causes loss of mobility. In contrast, insertion of COOH groups in compounds that are mobile in xylem, convert them in ambimobile.

2.2.3. Absorption of xenobiotics in the leaves

The epidermis of the leaf is not adapted to absorb dissolved substances. The presence of stomata and cuticle permit the regulation of gases exchange preventing the loss of water. The cuticle covers the leaves as a thick layer of 0.5 – 14 μm width, consisting of cutin, intracuticular lipids, polysaccharides and phenols. Epicuticular waxes impede adhesion of the compounds suspended in water on the surface of leaves. However the main barriers to penetration of polar xenobiotics are the intracuticular lipids. Non-dissociated molecules of acidic compounds enter preferentially with regard to the charged molecules. Additionally, intracuticular lipids retain lipophilic xenobiotics, providing in some cases a cumulative effect. Entry of compounds by abaxial side of leaves is more effective than by adaxial side.

In foliar applications, once the systemic fungicide has penetrated the cuticle, the movement of active ingredients is governed by similar factors. The xenobiotic can be translocated acropetally in the transpiration stream or accumulated in the complex of cribs tubes - companion cells. In general, it is agreed that sugars in the phloem produce an osmotic influx of water and the subsequent hydrostatic pressure move assimilates and xenobiotics to the accumulation organs.

3. RESISTANCE TO FUNGICIDES

3.1. Concepts and terminology

Organisms that exhibit a reduced sensitivity or insensitivity to a toxic sustance are termed as being "***resistant"***. In this concept, it is necessary to distinguish ***natural resistance*** in the total population of species, families etc. of fungi, from the ***acquired resistance*** by strains of a species that is normally sensitive. The latter constitutes the object of the present manual. It should be differentiated from resistance by adaptation or ***"training"*** that can be obtained when a culture *in vitro* of a microorganism is subjected to successive increasing rates of a xenobiotic, but that disappears when it is cultured again in a toxin-free culture media.

Resistance is a stable and inherited property of a pathogen and exhibits significantly reduced sensitivity compared to the sensitivity of normal population of the species or ***wilt type*** prior to being subjected to a particular fungicide treatment. Once established, resistance can remain for a prolonged period of time or disappear rapidly in the population. It is related to the **selective action** of systemic fungicides , especially biochemistry selective action (Leroux, 1987).

Individual resistance **(**also called ***laboratory resistance***) pertains to the resistance of an individual, clone, isolate or race that, under controlled conditions are inhibited by higher fungicide concentrations than those which in similar conditions affect the sensitive individuals of the same fungal species. Most specialists agree that individual resistance is genetically controlled. It is characterized by the level (amount of active ingredient tolerated by the tolerant culture compared to the original sensitive wild type).

Adaptability or fitness is a comparative concept. Some of the parameters can be studied *in vitro*; others only in the field. The properties that can be studied *in vitro* are germination, mycelial growth and sporulation in liquid media and on agar.

Properties that can be studied *in vivo* are the probability of infection, speed of colonization and sporulation in the host plants. Resistant mutants frequently have a reduced adaptability or fitness. If however some of these mutants, only a few cells, have a high degree of fitness compared to the sensitive strain, a resistant population can be established, under the selection pressure established by the fungicide. Studies must be carried out to test the adaptability between resistant and sensitive strains.

Resistance of population. In nature, a population will be considered resistant when there is a significant loss in the efficacy of a correctly-applied treatment, caused by the presence of individuals that are totally or partially resistant to the applied substance.

The resistance to a fungicide is expressed in the field when the frequency of resistant individuals and their resistance levels are sufficiently high.

If we review the simplest case in which the population of a pathogen is composed of two sub-populations, one sensitive and the other resistant, results that generally efficacy of the fungicides are compromised if a few percentage-points of the frequency of resistant individuals is recorded. In other words, over of a critical threshold, the population is considered to be resistant. As this value is frequently the limit of detection of resistance in nature, it implies that there generally exists a coincidence between detection of first resistant isolates and first observation in the reduction of efficacy of treatments. It is for this reason that in practice, resistance is already severe by the time it is detected. In fact, when populations have a few resistant individuals, it is almost impossible to detect them. It is for this reason that different mathematical models have been proposed to simulate the frequency of resistant individuals in the population.

Base line. The word 'baseline' has many uses in everyday language but all of them include the concept that it is a point of reference to be used in a decision making process. For fungicide resistance research and management a 'baseline' can be defined as (Russell, 2003):

"*A profile of the sensitivity of the target fungus to the fungicide constructed by using biological or molecular biological techniques to assess the response of previously unexposed fungal individuals or populations to the fungicide*".

The primary use of baselines is as a tool for the establishment of, and subsequent monitoring of, fungicide resistance management strategies. The term baseline is universally applied to new compounds obtained from new chemistry but when applied to molecules for which, for whatever reason, it is not possible to find a population that has never been exposed to the type of chemistry associated with the new molecule, the terms '*sensitivity profile*' or even '*pre-market introduction profile*' may often be used. For the purpose of this document, the terms are interchangeable, depending on the circumstances.

By implicit definition, the baseline is not a single data point but is constructed by sampling a number of individuals or populations and establishing the variability between them. The baseline, however expressed, visually, mathematically or both, will illustrate this variability.

For practical purposes, the baseline establishes an accepted reference point for sensitivity of a fungus to a fungicide. Fungal isolates or populations found to have a sensitivity profile that falls outside the baseline response are normally considered to be 'less sensitive' or 'resistant' to the fungicide.

3.2. Microbiological balance and selectivity

Plants are constantly in contact with many microorganisms but they are only attacked by a few. So susceptibility is generally the exception and resistance the rule. Many microorganisms

however can compete with pathogens for space and nutrients or interfere with them by antibiosis. In the soil, as in the aerial parts of plants, there are some very complex interrelations termed 'microbiological balance'. This balance can be seriously disturbed by the use of a selective fungicide that kills a part of a microbiological community and leaves the rest alive. This can have phytopathological consequences. Potential pathogens under certain conditions are slightly harmful to plants due to antagonism with other members of the same flora. If a selective fungicide is applied, potential pathogens that are relatively insensitive to this fungicide can cause severe damage. Bollen (1981) observed this effect with respect to *Rhizoctonia solani* in cereal with benomyl treatments which caused rupture in the microbiological balance of the soil. The changes of the microbiological balance are only due to elimination or reduction of certain sensitive fungi and the capacity to produce antibiotics and other secondary metabolites with a selective product.

3.3. Genetic and biochemical bases of resistance

Acquired resistance and tolerance to fungicides can be related also to the selective action that fungicides pose, especially biochemical selective action (Dekker, 1981). Until the 1970s, the development of resistance was a little known phenomenon. Compounds of copper and sulfur are known from the beginning of plant pathology as a science, while mercury derivatives are known for their fungicidal properties for more than 80 years. Even when it was possible to adapt fungi under laboratory conditions (training) to higher concentrations of these fungicides, resistance phenomena were not found in practice.

The phenomenon that specific fungicides affect a few genes, was what made resistance population outbreaks a possibility, because the mutation was sufficient to absolutely or partially lose the toxic effect to the cell (Georgopulos, 1981). This, to a large extent, is determined by the number of genes involved in the process and their mutability at the specific locus. As most fungi have high multiplication frequencies and produce thousand of spores in a short time, the possibility of rapid establishment of resistant populations can be understood.

Multisite fungicides (in general contact fungicides) interfere in several sites at the biochemical and genetic levels. More mutations need to occur simultaneously for the resistance appears and at the same time without affecting the adaptability and competitiveness with the wild type, something which has a low probability of occurring. These can explain the fact that resistance cases to multisite compounds are not known, in spite of their continuous use over many years. Thus, as the action mechanisms of a fungicide involve more biochemical action sites and more genes controlling the processes the probability of the establishment of viable and competitive resistant populations in the field is reduced.

In order to determine the types of resistance mechanisms, it is necessary to know the mechanism of action of the fungicide. The toxicity of various biochemical, specific fungicides is based on interference with a variety of cellular activities that include generation of energy, nucleic acid protein, sterols and phospholipid synthesis; mitosis, cell-wall synthesis, etc. and fungal secondary metabolism involved in pathogenicity (Sisler, 1988). Resistance mechanism can operate in different ways and are classified in several Types as follows:

Type I. Reduction of membrane sensitivity (shown in Blasticidin S in *Pyricularia*, etc)

Type II a. Increase of detoxification (identified for benzimidazole in *Verticillium;* PCNB in *Botrytis* etc).

Type II b. Reduction of the conversion of compounds to fungitoxic (Observed for 6-azauracil in *Cladosporium*).

Type III. Reduction of affinity with the site of action (observed in benzimidazoles and QoI's in different species of fungi (Table 2 and Table 7 respectively; carboximides in *Ustilago*, kasugamycin in *Pyricularia*;

Type IV a. Circumvention (as in case de *Ustilago maydis* at antimicyn A)

Type IV b. Compensation

Type V. Active elimination by active efflux (depends on energy) determining that site of action do not saturate with the toxin (shown in some IBE).

When resistance results in the presence of DNA (dioxyribonucleic acid) in genes of the cell chromosomes, it is termed ***chromosomic resistance (or nuclear resistance)***, which is quantitatively inherited in accordance with Mendel's laws. The **extrachromosomic resistance (or cytoplasmic resistance)** occurs when the DNA is in the gene distributed in cytoplasm (plasmids) or included in cellular organelles (mitochondria). In many cases a gene confers resistance to a fungicide but many times this character is linked to the process controlled by several genes (polygenic resistance).

Resistant mutants are described by their resistance level that can be defined by the magnitude or mutational change of sensitivity.

Resistance mechanisms to fungicides of different chemical groups are described in Table 1.

TABLE 1. Resistance mechanism of different fungicide groups

Fungicide or fungicide Class	**Mechanism of acquired resistance**
Aromatics hydrocarbures	Unknown. Cross resistance with dicarboximides.
Benzimidazole	Alteration of site of action (β tubulina)
Kasugamycin	Change of site of action (in ribosomes)
Phosfothiolates	Metabolic detoxification
Phenylamides	Alteration of site of action (RNA polymerase)
Dicarboximides	Unknown. Cross resistance with aromatic hydrocarbures
DMI's	Increment of efflux;
	Altered site of action (cytocrome P450);
	Altered demand of product in the site of action
	Over production of the site of action
Carboxanilides	Altered site of action (succinato-ubiquinone –oxidoreductase)
Strobilurines	Altered site of action (site in the complex bc1 in mitochondria)

***Cross resistance*.** When the fungicide efficacy fails due to development of resistance in a given organism, it is important to know if efficacy to other fungicides has also been affected. If a mutation affects sensitivity to one fungicide and not to other, then the two components are termed 'non-correlated' in terms of cross resistance. A **positive cross resistance** exists when a mutation results in a phenotype that is less sensitive than the wild type race to two different

fungicides. If a mutation results in a phenotype more resistant than the wild type to a chemical and more sensitive to other, then this is termed **cross resistance negative correlated** or simply **negative cross resistance** (Georgopoulos, 1981). In genetic terms, in positive cross resistance, the same mutation determines insensitivity or resistance to both toxics, meanwhile in the negative cross related resistance, the mutation determines the sensitivity to one toxic and resistance to other. However, one race can be resistant to two different fungicides due to two independent mutations and in this case are not involved in correlated resistance.

The mutants of many fungi are obtained by selection to a fungicide, i.e. aromatic hydrocarbures, are also resistant to the rest of the members of the group or to other groups members as there are benzimidazole and dicarboximide derivatives. This phenomenon occurs in a similar way with fungicides of benzimidazole and thiophanate, dicarboximide, phenylamide and in DMI's groups.

3.4. Dynamics of selection towards resistance - epidemiological bases of resistance

The populations of a target pathogen of fungicide treatments are sensitive to new effective active ingredients when introduced, in spite of the fact that pathogen sensitivity range in the population at the time of introduction is usually not known. It is possible that the organism is incapable of coping with the fungicide; the system required for this is beyond of its metabolic capability. On the other hand, there are mutants which are resistant in major or minor grade. The potential ceiling can be moderate or high.

The nature and the effectiveness of the fungicide are important to determine the response of the pathogen. The resistance to a site specific compound can involve one or more genes of the pathogen whose capacity of modification is unpredictable. The site specific fungicides are usually systemics and persistent and these properties made them highly effective, but also promotes a high degree of selection for resistance by maximizing exposure of the fungicide to the pathogen. Resistance of pathogens to multisite fungicide may also involve one or more pathogen genes and the ease of modification of each gene is unpredictable. Multisite compounds are usually non-systemic and lack persistence, so that they have lower effectiveness, which does not give an intense selection pressure for resistance.

Initially, resistance forms are probably at a selective disadvantage and survive only at low frequencies in the population; they have low fitness prior to the introduction of the fungicide. It is therefore necessary to monitor the population for this character from the earliest possible steps. Unfortunately monitoring suffers from two defects:

- The majority of the methods used detect only relative frequent mutants; they are often less effective than trial plots in detecting the more important, but less common, forms. This problem is illustrated in Table 2, which shows the sample size required to obtain with 95% of probability at least one of those mutant that occurs at low frequency. The figures are obtained from the binomial expression sample **size=log (1-p)/log (1-y)** where p is the probability required and y is the frequency.
- The second problem in monitoring is related to the condition of the first–found resistant mutants. Tests often indicate that they do not grow well on treated host plants, and that they are less fit on untreated plants than the common strains. Unfortunately, once observed these characteristics are often regarded as static. They may remain so, but if selection for resistance is maintained or increased, then genotypes with improved performance, both in treated and untreated host plants may emerge. Because of the

difficulties of adequate monitoring, the development of such changes may remain undetected for a considerable period of time.

TABLE 2. Sample size and sampling area required to obtain, with 95% probability, an unusual mutant occurring at low frequency in a pathogen population; sampling area required refers to the area per day of cereal crop receiving spores of *Erysiphe graminis*.

Mutant frequency	***Sample size required***	***Sample area required (ha/d)***
10^{-3}	3×10^{3}	0.2
10^{-5}	3×10^{5}	23.1
10^{-6}	3×10^{6}	230.8
10^{-8}	3×10^{9}	23 077.0

Given that the fungicide-resistant mutants do occur in pathogen populations not yet subjected to fungicide treatments, we need to understand the process that causes an increase in frequency of these mutants when the fungicide is used. Mather (1953) defined three classes of selection acting on populations of organisms, namely stabilizing, directional and disruptive (Figure 10).

a) ***Stabilizing selection*** is the process that maintains a high frequency of optimal phenotypes, i.e., those that best fit the usual range of circumstances that the population encounters. Environmental variations and production of similar phenotypes by different genotypes will result in genetic variability even in a population subject to a strong stabilizing selection.

b) ***Directional selection*** occurs if the average genotype in the population is not close to optimum; then selection will favor those phenotypes and their associated genotypes that are optimum; selection will skewed towards the new optimum. Directional selection is a gradual positive response to changes in the environment. It is of special importance in the context of fungicide resistance when the metabolic process is polygenic controlled and quantitative.

c) ***Disruptive selection*** occurs when two different environments are created that each favor distinct optimal characteristic. Selection for the two distinct optima may cause a break in the continuity of the population variation. Populations then show a bimodal frequency of resistant and sensitive individuals, and stabilizing selection will again occur, to maintain the selected phenotypes and their new respective optima. This is frequent in the case of resistance due to change of site of action controlled by a single pair of genes as is usually the case with pathogen populations treated with benzimidazole, and QoI's fungicides.

All three types of selection may occur simultaneously and to varying degrees in response to the environmental change caused by the introduction of a fungicide to control the pathogen. The relative importance of each will depend on the degree of use of the fungicide and the extent to which different mechanisms of resistance are available in the organism. One critical factor is the relation between the length of the reproductive cycle of the organism and the time during which the fungicide is active. If an organism has a long cycle relative to the duration of fungicidal activity, the population will change slowly if at all. Conversely if the pathogen has a short cycle relative to the duration of fungicide activity, there will tend to be more rapid population response, and the population will tend to follow up any change in fungicide use.

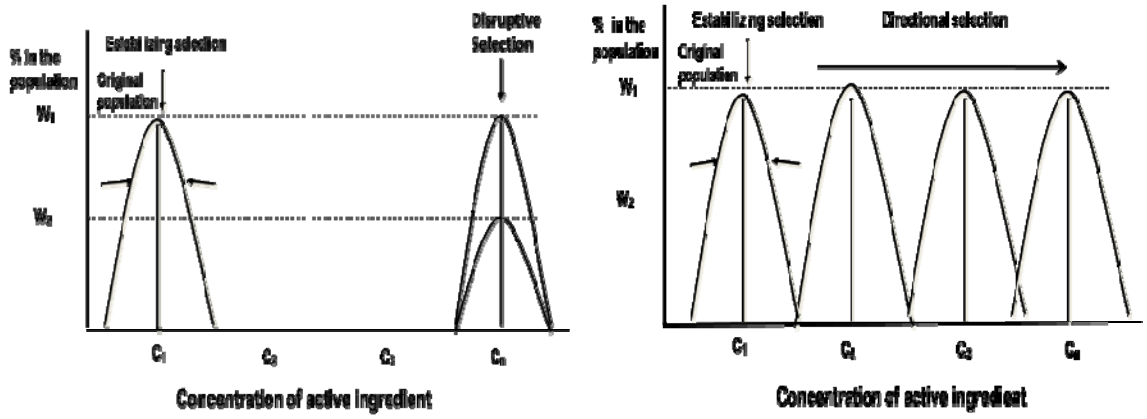


Figure 10. Stabilizing, disruptive and directional selection (Mather 1953; Wolfe, 1982).

When failures occur in the control of a disease after repeated applications of a fungicide that was originally effective, laboratory experiments can reveal when this phenomena is due to development of resistance to the compound. *In-vitro* emergence of resistance to a particular fungicide does not imply that the field use of the compound will lead to field failures of the disease control.

In nature, the first resistant individuals in the population can pre-exist or appear soon after of start of fungicide use. It depends of several factors; among them, the way of fungicide is used and the adaptability of resistant mutants with regard to wild type population. Resistant cases can develop spontaneously or resulting from of an induction by the fungicide. The latter possibility should not be excluded because some fungicides are mutagenic to fungi (benzimidazole and thiophanate, cyclic imides). Also, resistant isolates can be transported from a crop, a region or one field to other. It is probably this form of resistant isolates that are sometimes detected in zones where never the fungicide has been applied. Anyway, whatever the origin, these resistant isolates will initially be few in the population of the fungus. The phenomenon is similar to the presence of a single resistant individual in a population of a million ($1x10^{-6)}$ or a thousand million individuals (1×10^{-9}). Evidently at such low frequency, the treatments will continue be highly efficient.

The application of a fungicide destroys a large amount of sensitive individuals while the majority of the resistant individuals survive. Under these conditions, in each generation, the frequency of resistant individuals will continue to grow until it is high enough (several tenths of percentage) to cause a reduction or even a total loss of fungicide efficacy. From the epidemiological point of view, the emergence of resistant populations will depend on different factors as:

Fixed factors. (Biology of the fungus, active ingredient of fungicide and its biochemical characteristics)

- Mode of action (number and nature of the biochemical objectives that are inhibiting).
- Intensity of the reproduction of the objective pathogen.
- Epidemiology (length of periods of high pressure of infection)

Changeable factors (Use of the fungicide and other measures of control).

- Duration of exposure to the fungicide.
- Number of generations of the pathogen during exposure.

- Presence of other control methods (mixtures of products of different biochemical mechanism of action; sanitation; etc.)
- Size of original population under treatments.
- Size of the treated area in relation to untreated with the product, proportion of treated area with regard to the untreated or proportion of the population subjected to selection.
- Total or partial mortality of the population with the treatments.

In practice, favorable conditions for the fast development of fungicide resistance include:

- Fungi that attack aerial parts (leaves/stems) with short generation span, which produce many dispersible spores.
- Climatic and cultural factors favorable to fungal diseases (i.e. unbalanced fertilization, varieties very sensitive to parasites, very favorable weather, etc).
- Use of very effective monosite fungicides to which resistant isolates can be very easily developed in nature.
- Intensive utilization in time and space of the same risky fungicide

4. MAIN ACTIVE INGREDIENT FAMILIES OF SYSTEMIC FUNGICIDES USED IN BANANA PRODUCTION

The classification of fungicides based on the mode of action (MOA) as determined by the Fungicide Resistance Action Committee (FRAC, 2007) is shown in Table 3.

4.1. Methyl Benzimidazole Carbamates(Frac Code B1)

In this group are included benomyl, thiabendazole, carbendazim (MBC) and thiophanate methyl among others (Figure 10). Thiophanates are not benzimidazole until metabolized in the fungal cell. All the members in aqueous dilution are transformed to MBC or carbendazim, which is the true fungitoxic agent. From a taxonomic point of view and use, all have a wide action spectrum (with the exception of oomycetes), and are widely used for the control of foliar diseases in horticultural, fruits and cereal crops in postharvest and seed treatments alone or in mixtures with other active ingredients. Rates of use are in the range of 150 and 250 g ai./ha. In postharvest treatments concentrations are between 250 a 500 µg/ml (ppm).

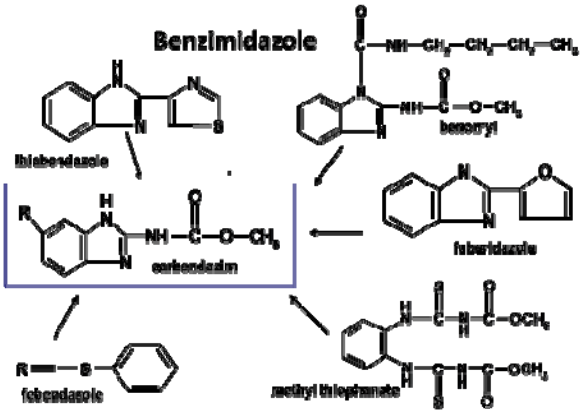


Figure 10. Benzimidazole fungicide group members

TABLE 3. List of chemical groups of fungicides used on banana, arranged by FRAC Code, based on mode of action (FRAC, 2007).

CODE	TARGET SITE	GROUP NAME	CHEMICAL GROUP	COMMON NAME	COMMENTS
B	**MITOSIS AND CELL DIVISION**				
B1 #1	mitosis: ß-tubuline assembly	**MBC** - fungicides (**M**ethyl **B**enzimidazole **C**arbamates)	benzimidazoles	benomyl carbendazim fuberidazole thiabendazole	- Resistance common in many fungal species. - Several target site mutations, mostly E198A/G/K, F200Y - Positive cross resistance between the group members. Negative cross resistance to N-Phenylcarbamates - **High risk.**
			thiophanates	thiophanate thiophanate-methyl	
B2 #10	mitosis: ß-tubulin assembly	N-phenyl carbamates		diethofencarb	- Resistance known. Target site mutation E198K. - Negative cross resistance to benzimidazoles. - **High risk.**
C:	**RESPIRATION**				
C3 #11.	Inhibition of complex III of fungal respiration: at Cytochrome bc1 (ubiquinol oxidase)	**QoI**-fungicides Qo site (**Q**uinone **o**utside **I**nhibitors)	methoxyacrylates	azoxystrobin picoxystrobin	- Resistance known in various fungal species. Target site mutations G143A, F129L and additional mechanisms. - Cross resistance shown between all members of the QoI group. - **High risk.**
			methoxycarbamates	pyraclostrobin	
			oximino-acetates	kresoxim-methyl trifloxystrobin	
			oximinoacetamides	metominostrobin	
			oxazolidinediones	famoxadone	
			dihydrodioxazines	fluoxastrobin	
			imidazolinones	fenamidone	
			pyridine carboxamides	boscalid	
D:	**Amino Acid and Protein Synthesis**				
D1 #9	methionine biosynthesis (cgs gene proposed)	AP - fungicides (Anilino-Pyrimidines)		cyprodinil mepanipyrim pyrimethanil	- Mechanism speculative (CGS). - Resistance known in Botrytis and sporadically in Venturia - Medium risk

CODE	TARGET SITE	GROUP NAME	CHEMICAL GP	COMMON NAME	COMMENTS
G:	**Sterol Biosynthesis Inhibitors (SBI fungicides)**				
G1 **#3**	C14-demethylation in sterol biosynthesis (erg11/cyp51)	(SBI: Class I) **DMI**-fungicides (**D**e **M**ethylation **I**nhibitors)	imidazoles	imazalil pefurazoate prochloraz triflumizole	- There are great differences in the activity spectra of the different DMI fungicides. - Resistance is known in various fungal species. Several resistance mechanisms known including target site mutation Y136F (erg11/cyp51), ABC transporters (efflux) and others. - Generally wise to accept that cross resistance is present between fungicides active against the same fungus. - DMI fungicides are Sterol Biosynthesis Inhibitors (SBI's) but show no cross resistance to other SBI classes. - **Medium risk.**
			piperazines	triforine	
			pyridines	pyrifenox	
			pyrimidines	fenarimol nuarimol	
			triazoles	azaconazole bitertanol bromuconazole cyproconazole difenoconazole diniconazole epoxiconazole fenbuconazole fluquinconazole flusilazole, flutriafol hexaconazole imibenconazole ipconazole, metconazole myclobutanil penconazole propiconazole prothioconazole simeconazole tebuconazole tetraconazole triadimefon, triadimenol triticonazole	

CODE	TARGET SITE	GROUP NAME	CHEMICAL GP	COMMON NAME	COMMENTS
G:	**Sterol Biosynthesis Inhibitors (SBI fungicides)**				
G2 **#5**	ΔC^{14}-reductase and ΔC^{8}-ΔC^{7} isomerase in sterol biosynthesis	(SBI: Class II) **Amines: ("Morpholines")**	morpholines	**aldimorph** dodemorph fenpropimorph tridemorph	1. **Decreased sensitivity described for** 2. powdery mildews. 3. Cross resistance within the group generally found but not to other SBI classes. 4. **Low to medium risk.**

CODE	TARGET SITE	GROUP NAME	CHEMICAL GROUP	COMMON NAME	COMMENTS
P	**Host defense inducer**				
P1: **# P:**	salicylic acid pathway host plant defense induction	benzo-thiadiazole BTH		acibenzolar-S-methyl ASM	5. Resistance not known
M:	**Multi Site Action**				
	SH^{-} groups in respiration enzymes	dithiocarbamates & relatives		mancozeb zineb propineb	No resistance cases
		chloronitriles		chlorothalonil	No resistance cases

Benzimidazole and thiophanate are systemic fungicides that block microtubules assembling (Figure 11) when selectively joined with fungal β tubuline (not in mammals) impeding the coupling of dimmers of α y β tubulins, and hence cellular mitosis (Davidse, 1981). Benzimidazole treated spores of sensitive populations exhibit short and deformed germ tubes, or do not germinate (Figure 12; Davidse 1980, Pérez y Mauri 1981).

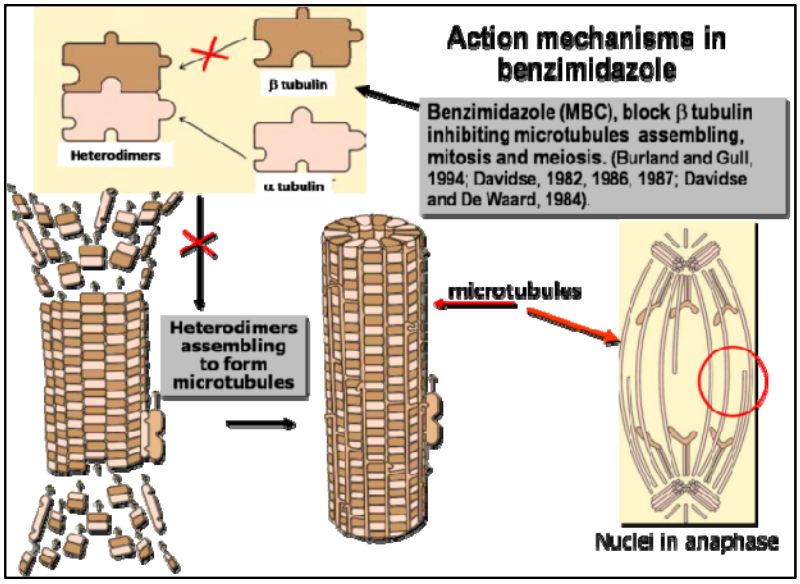


Figure 11. Mechanism of action of benzimidazole. Benzimidazole couples with ß tubuline impeding the assembling of heterodimers, the microtubules developing and hence, mitosis.

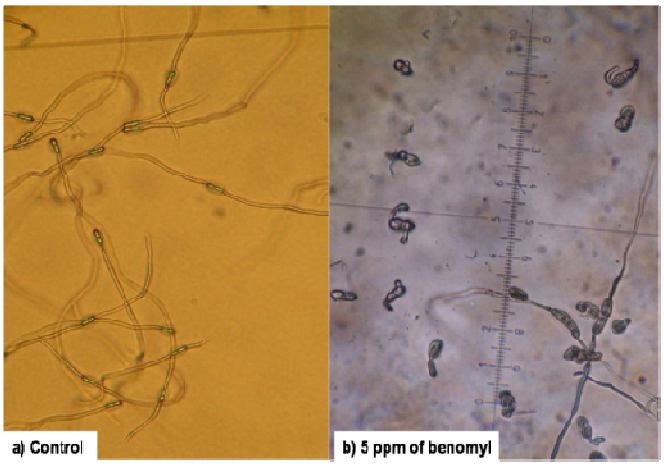


Figure 12. Ascospores germinated in water agar: a) normal germination in agar without fungicide; b) distorted germ tubes in a media amended with 5 µg/ml of benomyl.

As the process is governed by a single pair of genes, risk of selection of tolerant populations are high, as has been shown in the existing global records of many crops. The population shows a bimodal curve in terms of sensitivity with two different subpopulations, one sensitive and other highly resistant with different levels of fitness. Selection is then disruptive and the tests for monitoring are qualitative, with a procedure based on threshold concentration.

Use of benzimidazole and thiophanate methyl in Sigatoka leaf spots management. For Sigatoka leaf spot management a maximum of two or three treatments per cycle of benomyl (150 g de a.i./ha), carbendazim (200 g a.i./ha), and methyl thiophanate (280 g

a.i./ha) should be used in mixtures with mineral oil and a multisite inhibitor fungicide (Stover, 1980, 1990; Pérez, 1978; Pérez, 1978; Pérez *et al*. 1993; Pérez and Mauri, 1994). In presence of sensitive populations, these exhibit high efficacy and adapt perfectly to bioclimatic warnings for treatments. Benzimidazole show negative cross resistance with N phenylcarbamates. Resistant strains of *M. fijiensis* and *M. musicola* show a high fitness and spread fast. Resistance can be stable for a long time after withdrawing benzimidazole from management strategy. In most banana producing areas, there exists high level of resistance in *Mycosphaerella* populations. Davidse and Ishii (1995) reviewed the single aminoacid changes leading to loss of affinity between benzimidazole molecule and the β tubulin in different species of fungi (Table 4). TBZ and carbendazim has been widely used for crown rot (fungal complex of *Fusarium pallidoroseum*, *Colletotrichum musae*, and other concomitant fungi) and anthracnose disease (caused by *Colletotrichum musae*) management in postharvest treatment by dipping, cascade or spraying of the crown at 400 μg/ml concentration.

TABLE 4. Amino acid substitution in β tubulins in laboratory mutants and strains with resistance to benzimidazole (according Davidse and Ichii, 1995).

Codon	Substitution	Organism
6	His for Leu	*A. nidulans* [a]
	His for Tir	*A. nidulans* [a] y *S. nodorum* [b]
50	Tir for Asn	*A. nidulans* [c]
	Tir for Ser	*A. nidulans* [c]
134	Gln for Lis	*A. nidulans* [c]
165	Ala for Val	*A. nidulans* [d]
167	Fen for Tir	*N. crassa* [e]
198	Glu for Ala	*B. cinerea* [f, g], *N. crassa* [b]; *P. aurantiogriseum* [i]; *P. expansum* [c]; *P. puberulum* [i]; *V. inaequalis* [c]
	Glu for Asp	*A. nidulans* [a]
	Glu for Gln	*A. nidulans* [a]
	Glu for Gly	*N. crassa* [b]; *R. secalis* [i]; *V. inaequalis* [i]
	Glu for Lis	*A. nidulans* [a]; *B. cinerea* [g]; *M. fructicola* [i]; *P. digitatum* [c]; *P. italicum* [c]; *R. secalis* [i]
	Glu for Val	*P. expansum* [c].
200	Phe for Tyr	*B. cinerea* [g]; *P. aurantiogriseum* [i]; *P. italicum* [i].
241	Arg for His	*S. saccharomyces* [j]
257	Met for Leu	*A. nidulans* [c]

Taken from Davidse and Ichii (1995) from: (a) Jung et al. (1992); (b) Cooley and Caten (1993); (c) Koenraadt et al. (1992); (d) Jung and Oakley (1990); (e) Orbach et al. (1986); (f) Martin et al. (1992); (g) Yarden y Katan (1993); (h) Fujimura et al. (1992); (i) Wheeler et al. (1994); (j) Thomas et al. (1985).

4.2. Sterol biosynthesis inhibitors (SBI)

Among the systemic fungicides, the inhibitors of ergosterol biosynthesis have a special importance for different reasons. These are not classified according a common structure but by their action mechanism. In this group a large number of diverse compounds that interfere with the *novo* biosynthesis of sterols are included. Not all the compounds are involved in phytopathogenic fungi control. Although ergosterol is the main sterol of most fungi, there are a lot of exceptions. Several powdery mildew fungi and rust fungi contain no ergosterol. The main sterol of powdery mildews was identified as ergosta-5,24(28)-dienol and stigmasta-7,24(28)-dienol (Loeffler *et al*., 1984; Jackson and Frear, 1968; Koeller, 1972; Kuck, et al., 1995). This means that the term "sterol biosynthesis inhibitors" have a wider connotation and are members of a group that include sterols, gibberelins, carotenoids, etc. Therefore the term "sterol biosynthesis inhibitor" (SBI) is more appropriate than "ergosterol biosynthesis inhibitor" (EBI).

SBI's have been one of the most important fungicide groups in agriculture. There are two SBI types (Köller y Scheinpflug 1987; Sisler 1996; Fig 13): DMI's or α C14 - demethylase inhibitors in lanosterol (SBI, inhibitors type I) and Δ8 - Δ7 isomerase/ Δ14 - Δ15 reductase inhibitors (SBI, type II). In medicine there is a third group: inhibitors of esqualene epoxydase.

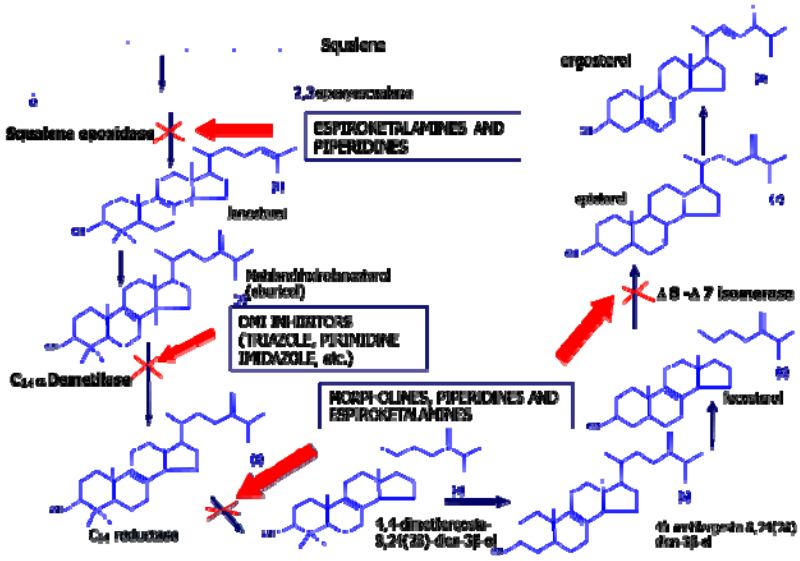


Figure13. Metabolic pathway of ergosterol byosinthesis and action points of different SBI's fungicides.

Their chemical and physico-chemical properties have an influence on their possibility to penetrate the plant and translocate through apoplast. All SBI's have the property to penetrate plant cuticle and/or seed envelop to some extent. After penetration, they can be translocated mainly via the apoplastic pathway. They also have secondary effects on plant growth such as shortening of internodes, darkening of green color, transpiration reduction, and resistance to different stress types.

The group is effective against ascomycetes, deuteromycetes and basidiomycetes. Almost all are active against powdery mildews and rusts to a certain degree which are harmful obligate parasites. Oomycetes and zygomycetes are not sensitive. These fungicides block the *novo* biosynthesis of ergosterol in the cell membrane. Spores germinate with the constitutive ergosterol present in the cell, so the main effect is on the germ tube and hyphal

growth. They also have a strong therapeutic effect on spore production on plant spots. Sisler (1996), review the main characteristics of these compounds in the following way:

4.2.1. SBI of type I or DMI's (α C14-demethylase inhibitors; FRAC Code G1)

This group includes triazole, pyrimidine, piperazine, pyridine, and imidazole chemical groups. Most common DMI's fungicides used in banana belong to triazole and imidazole chemical groups.

4.2.2. DMI's uses in disease control in banana and plantains

Among the DMIs, triazole (Figure 14) has shown the best efficacy level against yellow and black Sigatoka disease. DMI's cause inhibition of growth of *M. fijiensis* spore germ tube and production of fructification bodies (Pérez *et al.*, 1993; Pérez and Mauri, 1994). In this group are products differing in degree of translaminar and systemic movement and efficacy on different fungal taxonomic groups. Most common used fungicide of the group for BSD management have been (Figure 14), propiconazole, tebuconazole, triadimenol, hexaconazole, fluzilazole, difenoconazole at the rate of 100 g a.i./ha; cyproconazole and epoxyconazole at rates of 80 g de a.i. /ha. The most widely studied and used triazole in banana has been propiconazole.

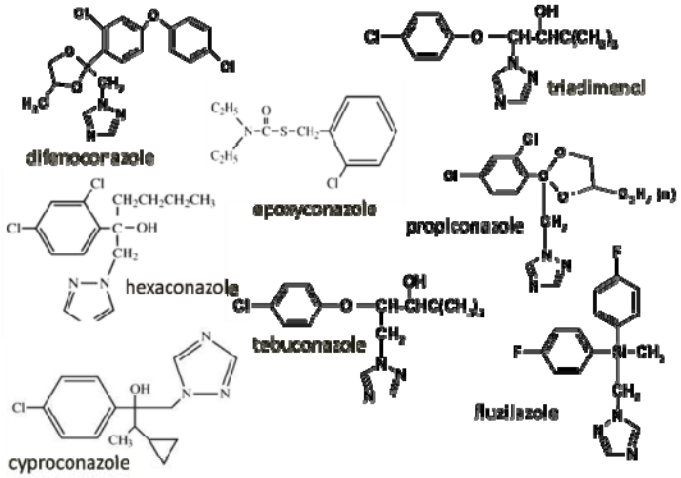


Figure 14. Most common triazole fungicides used in Sigatoka leaf spots management

The recommendation is to use no more than four to six treatments/year of triazole in oil mixture or emulsions of fungicide (80-100 g a.i.) + oil (7-10 L) + an emulsifier + water (to complete 20-25 L/ha of final solution).

Propiconazole has been used In Cuba since 1983 and lost of sensitivity of *M. musicola* populations (Muiño *et al.* 1987) and *M. fijiensis* (Pérez *et al.,* 2003) have been observed. ED_{50} of propiconazole in wild *M. musicola* and *M. fijiensis* strains have been estimated in 0.003 μg/ml. After several years of use, populations with a resistance factor (RF) of 10 have been found (Pérez *et al.,* 2003). In Central America due to an excessive use of triazole fungicides populations with RF of 100, have been selected leading to a loss of efficacy and extensive losses by BSD.

Imidazole fungicides imazalil and prochloraz (Figure 15.) are used in the control of crown rot disease at the rate of 250-300 μg/ml in postharvest treatments.

DMI's are considered fungicides with a moderate risk of resistance that develop resistance gradually (directional selection) and the loss of activity is considered transient. In these

fungicides, resistance is controlled by several genes (Buchenauer, 1995). Mutations in a single gene usually result in a low degree of resistance and resistance levels may increase by additional mutations of other genes (multistep mutations).

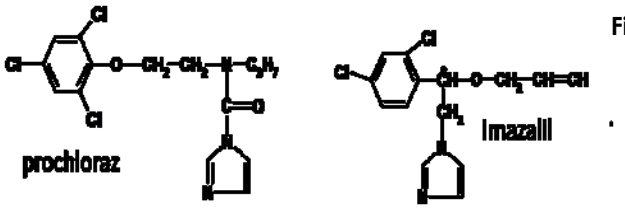


Figure 15. Imidazole (DMI's) fungicides used in post harvest treatment on banana for crown rot and anthracnose fruit diseases.

Three main molecular mechanisms have been described as responsible for the appearance of resistance to DMI fungicides in other fungi: point mutations in the coding region of the sterol 14*α*-demethylase gene, (Balashov *et al.,* 2005; Wyan *et al.,* 2005) protein overexpression11,12 and over expression of ATP-binding cassette (ABC) transporters encoding efflux pumps.13,14

Fraije *et al.* (2007) implicated alterations in the target-encoding sterol α C14 -demethylase protein (CYP51), and over-expression of genes encoding efflux pumps, in reducing sensitivity to the azole class of sterol demethylation inhibitors (DMIs), as well as the prevalence and selection of two CYP51 alterations, substitution I381V and deletion of codons 459 and 460 (ΔY459/G460), in populations of *Mycosphaerella graminicola*. Data linking fungicide sensitivity with the presence of I381V in *M. graminicola* show for the first time that a particular CYP51alteration is differentially selected by different azoles in field populations of a plant pathogen and that the use of different DMI's with negative cross resistance can be included in an anti resistance strategy.

Cañas-Gutiérrez *et al.* (2009), identified six mutations at CYP 51 (Y136F, A313G, Y461D, Y463D, Y463H and Y463N) that could be related to sensitivity loss to propiconazole in *M. fijiensis* isolates.

As the process is by multistep mutations, the selection process is then directional and monitoring procedure is quantitative. As previously explained, DMI's block the *novo* synthesis of sterols and spores germinate with constitutive ergosterol present in cell, so the main effect is on the germ tube and hyphal growth. Hence, monitoring sensitivity tests are based on inhibition of ascospore germ tube growth at different concentrations. Analysis involve the frequency of spores in different ranges of inhibition related to a wild type population or a base line of frequency of ascospores germ tube inhibition in a given site under study.

4.2.3. SBI of Group II (Inhibitors of Δ8 - Δ7 isomerase/Δ14-Δ15 reductase. Frac Code G5 "amines")

Fungicides inhibitors of Δ8-Δ7 isomerase /Δ14-Δ15 reductase have a nitrogenated heterocycle that is a morpholine or a piperidine and can be considered multisite inhibitors.

*Morpholine***:** Tridemorph, fenpropimorph (Figure 16) are active ingredients developed for the control of powdery mildews in cereals. The first investigations on systemic properties of morpholine derivatives were carried out with tridemorph. Soil treatment experiments with barley plants readily took up tridemorph by roots and transported it acropetally in the transpiration stream into leaves and 48 hours after treatment equilibrium had been established between a nutrient solution with 50 ppm of triadimenol and leaf extracts of plants. Absorption of the chemical by leaves occurred more slowly. After leaf penetration, transport occurred rapidly (Pommer *et al.,* 1969).

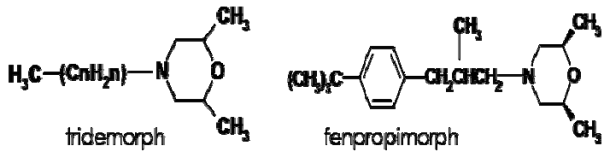


Figure16. Morpholine fungicides used in Sigatoka leaf spots control.

Spiroketalamines. The only molecule of this group is spiroxamine (Figure 17) which contains four enantiomers (Table 6), that contribute in different degrees to the complete biological activity against fungi.

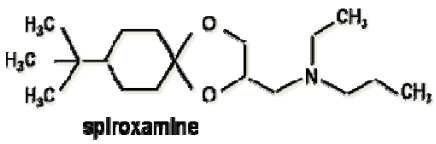


Fig. 17. Structural molecule of the amine fungicide spiroxamine.

Table 6. Point of action of enantiomers isomers of spiroxamine (from Bayer, 2012)

Point of inhibition	Isomers			
	GAW 295 (A/S)	GAW 296 (A/R)	GAW 297 (B/S)	GAW 298 (B/R)
Squalled epoxydase	X			
Epoxyscualen cyclase		X		
Sterol Δ C_{14} reductase	X	X	X	X
Sterol $\Delta C_8 \rightarrow \Delta C_7$ isomerase			X	X

Spiroxamine quickly penetrate foliar tissues. After ten minutes, approximately 1/3 of the a.i. penetrates inside leaf and after three hours the full active ingredient reaches the inside of the leaf. Translocation is limited and the chemical does not accumulate in the border of leaves.

4.2.4. Use of type II SBI's (amines) in the control of *Mycosphaerella* spp. in bananas

Morpholines are used in the range of 450 g a.i. /ha in 10 -12 l of mineral oil/ha. Tridemorph have shown problem of phytotoxicity in form of fruit speckling.

Spiroxamine. As indicated earlier, spiroxamine can be used at the rate of 320 g a.i. /ha in mixtures or emulsions with mineral oil and morpholine. After several years of use no loss of sensitivity has been found in *M. fijiensis* populations.

After more of 20 years of morpholine use, there are no records of loss of sensitivity, so this group has great importance in anti resistance strategy to avoid selection of resistant populations to other chemical groups.

As the process of resistance to amine is multistep, selection process is directional. Sensitivity monitoring analysis is quantitative, similar to that of DMI's based on inhibition of germ tubes at different concentrations of active ingredient and in frequency distributions of spores at different levels of inhibition related to a base line of reference or wild type isolates obtained on the site under study.

4.3. Qo's Inhibitors (Inhibition of complex III of fungal respiration at cytochrome bc1 (ubiquinol oxidase. FRAC code C3 # 11)

The development of this group of fungicides came from modeling the strobilurine A produced by *Strobilurus tenacellus* which are unstable in presence of light (Figure. 18.)

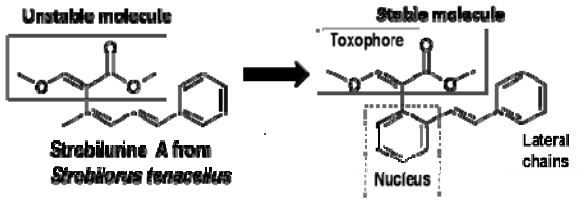


Figure 18. Unstable strobilurine A produced by *Strobilorus tenacellus* and stable derivative form developed from it.

The Qo's inhibitors are divided in two groups based on the coupling site in the mitochondrial membrane: QoI's are in the internal site and QoO's at the external site.

QoI's fungicides. The group is composed by seven chemical families of fungicides that share the same action mechanism. These are methoxyacrylates (azoxystrobin), methoxycarbamates (pyraclostrobin), oximino-acetates (trifloxystrobin), oximinoacetamides (metamidone), imidazolinones (fenamidone), oxazolidinediones (famoxadone), dihydrodioxazines (fluoxastrobin) and pyridine carboxamides (Figure 19).

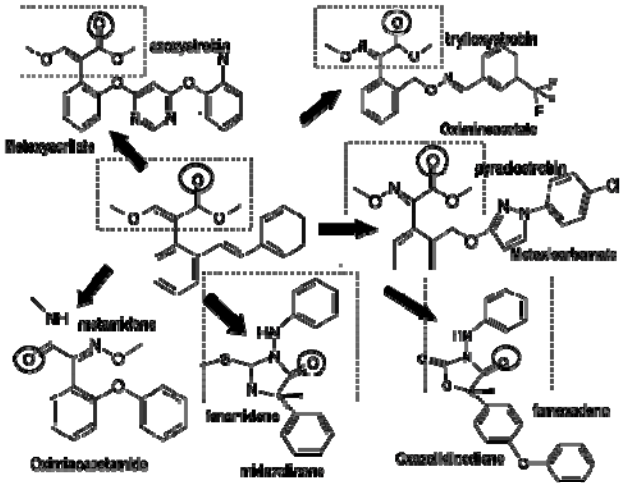


Figure 19. QoI's chemical families and most representative fungicide member of the chemical family

Most of them have strong systemic and translaminar action when applied to leaves which are variable for the different compounds. They inhibit germ tube growth and sporulation. In the group are compounds of a high efficacy against a wide taxonomic group of fungal species but at the same time of variable efficacy in dependence of the taxonomic group.

The mode of action of this group is the blocking of electron transport at bc1 cytochrome in the outside site of inner membrane of mitochondria inhibiting ATP synthesis (Geier *et al.*, 1992). These compounds have a wide and at the same time variable spectra of action against different taxonomic groups of fungi.

4.3.1. Mutations associated with QoI-resistance

Azoxytrobin and trifloxystrobin bind to Tyr 273 and Ala 127 aminoacids (Ziegler et al., 2003) of cytochrome bc_1 protein. Studies of mutagenic events to describe possible resistant mechanisms are recorded in Table 7. Two main amino acid substitutions have been detected in the cytochrome b gene in plant pathogens that govern resistance to Qo

inhibitors: the mutation G143A (change of glycine aminoacid by alanine at position 143) and change from phenylalanine to leucine at position 129 (F129L) have the higher practical importance (Sierotzki *et al*., 2000 a, and b; Heaney *et al*., 2000; Kuck and Mehl, 2003; Fig. 20).

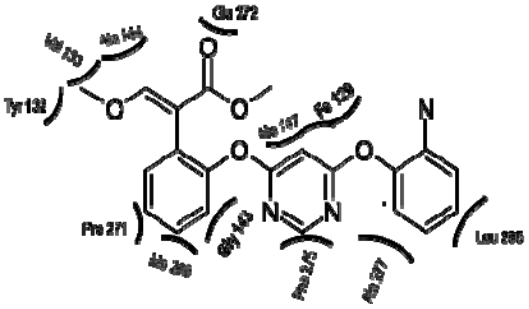


Figure 20. Azoxystrobin binding site at cytochrome bc1 (modified from Gisi *et al.*, 2002)

Table 7. Aminoacid substitutions in ubiquinol: cytocrome c oxide reductase (bc_1 complex), leading insensitivity to QoI's fungicides.

Aminoacid substitution	Species	Sources
Fen 129 by Leu	*Magnaporthe grisea*	Kim *et al*., (2003)
Asn 261 by Asp	*S. cerevisiae*	Köller, (1999)
Gly 143 by Ala	*M. fijiensis*	Sierotski *et al*. (2000)
	Mycena galoparda	
	Pseudoperonospora cubensis	
	M . grisea	Kim *et al.,* 2003
Ser 254	*Sch. pombe*	Zheng *et al.* (2000)
Pro 254 by Gln	*S. cerevisiae*	Joseph-Horne *et al.* (2001)
Gly 143 by Ser	*M. grisea*	Avila-Adame and Köller, (2003)

Both G143A and F129L are based on single nucleotide polymorphisms in the cytochrome b gene; the selection process is qualitative (single step).

Based on current knowledge, resistance factors (RF = ED50*[1] [resistant strain] / ED50 [sensitive wild-type strain]) associated with G143A and F129L are different. Resistance factors caused by F129L usually range between 10 and 50, whilst resistance factors related to G143A are in most cases greater than 100 (Table 8).

G143A has been shown to be responsible for QoI resistance in more pathogen species than F129L (17 out of 21 plant pathogens carry G143A or both of the mentioned point mutations in combination). Isolates carrying G143A express high (complete) resistance. Isolates with F129L express moderate (partial) resistance. F129L has been detected in 6 out of 21 plant pathogens, with 2 pathogens possessing both mutations.

4.3.2. Use QoI's in the control of Mycosphaerella spp. in bananas

For *Mycosphaerella* spp. control in banana, is recommended no more than two to three application per cycle of 100 g of a.i./ha of azoxystrobin and 80 -100 g a.i./ha of trifloxystrobin

[1] *(Effective dose 50): Effective dose with 50% response (inhibition)

in straight oil or water in oil emulsions in mixtures and/or rotation with fungicides of different chemical families and action mechanisms.

M. fijiensis populations with high levels of resistance to azoxystrobin, trifloxystrobin and other QoI's fungicides have been widely reported in Central America and Asia.

Table 8. Aminoacid changes in different positions of bc1 complex that conferring resistance to mixothiazol (QoI) in different species. (Data modified from Gisi *et al.*, 2002)

Aminoacid position and change	Species	RF
F129 → L	*Saccharomyces cerevisiae*	930
F129 → L	*Rhodobacter capsulatus*	500
F129 → L	*Chlamydomonas reinhardtii* (alga)	430
F129 → S	*Rhodobacter capsulatus*	930
G137 → R	*Saccharomyces cerevisiae*	4
G137 → E	*Saccharomyces cerevisiae*	20
G137 → V	*Saccharomyces cerevisiae*	4
G137 → S	*Saccharomyces cerevisiae*	37
G143 → A	*Mus musculus* (ratón)	2000
G143 → D	*Rhodobacter capsulatus*	10000
G137 → T	*Paramecium aurelia*	22000
G143 → A	*Paramecium aurelia*	22000
N256 → F	*Paramecium aurelia*	22000
N256 → Y	*Saccharomyces cerevisiae*	6
L275 → T	*Saccharomyces cerevisiae*	6
L295 → F	*Mus musculus*	4

4.4. Anilino-Pyrimidines (Inhibitors of methionine byosinthesis. Frac code D1 #9).

In this fungicide group are three active ingredients: cyprodinil, mepanipyrim and pyrimethanil. Figure 21 shows the pyrimethanil molecule. The action mechanisms of this group are different from the other fungicide groups. All are hydrophobic and of low toxicity to mammals. They inhibit the production of proteins and enzymes related to pathogenesis (Figure 22).

Pyrimethanil shows contact and translaminar preventive and therapeutic action in foliar treatments. It is taken up by roots in soil applications in vegetables and shows a good systemic movement. In foliar sprays the movement is basically translaminar and is based on vapor activity. It can control *Botrytis* isolates resistant to dicarboximide fungicides in grapes and other crops, and does not show cross resistance to benzimidazole, DMI's, amines, morpholines and dicarboximided and other fungicide groups used in banana.

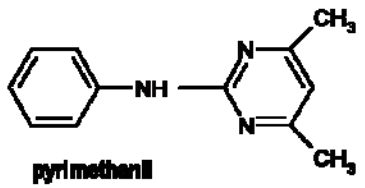


Figure 21. Molecule of pyrimethanil

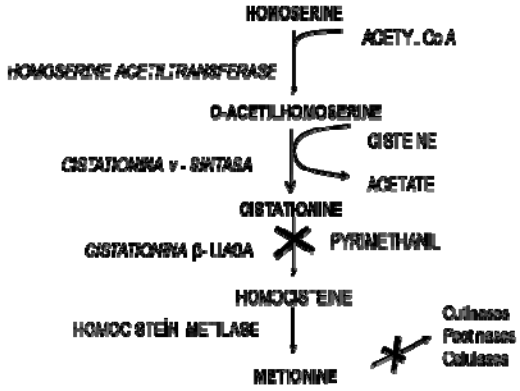


Figure 22. Simplified metabolic pathway of methionine biosynthesis. Mechanism of action of aniline pyrimidines. (Modified from: a. Masner, et al. Pest Sci. 42, 163-166 1994; b. Pontzen, 1997; c. Milling y Daniels, 1996; d. Dehne, 2002 In: Modern fungicides and antifungal compounds III, pp 141-148).

.

4.4.1. Use pyrimethanil in the control of *Mycosphaerella* spp. in bananas

In banana, pyrimethanil has been introduced in rotation to other fungicides in the control of *Mycosphaerella* leaf spots. For black Sigatoka control the use of 300 – 400 g a.i./ha in mixtures with 7-15 litres of mineral oil has been recommended. Pyrimethanil level of efficacy is similar to morpholines. After several years of use in different contexts, no loss of sensitivity of M. fijiensis has been reported.

4.5. Carbamates. Derivatives of dithiocarbamic acid

In this group are important active ingredients widely used in the control of plant diseases as mancozeb, maneb, zineb, propineb, TMTD, etc. All are contact fungicides that inhibit spore germination and sporulation when in contact with reproductive bodies of fungi. Their mechanism of action are based in the block of SH groups present in different enzymes of cell metabolism so are multisite compounds that do not develop resistance population. The fungicides are used in mixtures and alternance with unisite systemic compounds. Rates of use are from 1.5 - 2.5 kg. a.i./ha.

4.6. Chlorothalonil

This is a contact fungicide with a wide spectrum of action. The mechanism of action is related with the reaction with glutathion, coenzyme A, 2-mercaptoetanol and other compounds forming S derivatives, reducing the SH content on cells and inhibiting all reactions dependent of thiol groups (Vincent and Sisler, 1968). Glutathion is an important component of cell metabolism and is quickly affected by chlorothalonil. Their action is therefore unspecific and multisite. Chlorothanil has problems of toxicity to fish.

It is used at rate of 0.6 – 2.5 kg ia./ha in water for Sigatoka leaf spots control because it causes phytotoxicity when mixed with mineral oil. It has been used in rotation with unisite fungicides to reduce the selection of resistant populations.

5. PROTOCOLS FOR SAMPLING AND MONITORING SENSITIVITY OF *Mycosphaerella* spp. POPULATIONS TO BENZIMIDAZOLE, DMI'S, AMINES, QoI's AND AMINOPYRIMIDINES FUNGICIDES

In Annex 1, the most important equipment and materials required to conduct tests are listed.

5.1. General procedure for sampling

Sampling planning should be carried out taking into account different factors:

- History of frequency of fungicide use and efficacy of the treatments. It is very important to collect all relevant field information on efficacy and fungicide use with monitoring results that would enable complete data interpretation and drafting of conclusions on population sensitivity shifts.
- Lines of flight applications in the case of aerial treatments. Banana field sectors applied by aircrafts which are in pre-established lines of spraying, receive usually the same products in the same moment. So it is important to know the lines of flight and spraying to establish a best representative sampling of all the different areas of the plantation.
- Topography and obstacles to spraying. Irregular topography and obstacles have impact on efficacy of treatments. In irregular topography and close to obstacles, spraying height are variable and fungicide deposit irregular so efficacy is affected and can conduce to misleading conclusions regarding sensitivity shifting.
- Rainfall pattern on the site. Rainfall pattern is the most important factor on BSD development and spread. Banana fields in high rainfall areas are subject to high inoculum pressure, are more frequently treated with fungicides and selection pressure are more active. Such regions should be prioritized.
- Sanitation practices. Sanitation is important to reduce pathogen exposition to fungicides and shift in sensitivity selection pressure. Fields with poor sanitation practices are more prone to have shifts in *M. fijiensis* population sensitivity.
- Field configuration and extension. This is important to determine the number and places of leaf sampling.

Sampling points. Sampling points should represent conditions of production fields in the site. It is advisable to establish permanent sampling stations that provide the opportunity to determine year to year variations on population sensitivity to fungicides in use. Samples should be taken at least one time in a year. In sites with two well-defined seasons (rainy and dry) monitoring can be carried out at beginning and end of the rainy season. Monitoring at beginning of rainy season will provide some information on the status of sensitivity after several months of dry weather and allow comparison with results of the monitoring at end of previous rainy season. Monitoring at end the rainy season gives the most important information and assists with establishing fungicide use strategy for the next cycle.

Sampling procedure:

- Samples should be taken at least 8 days after the last spray of the fungicide that is to be monitored. Plants to be sampled should be adults, not flowered.
- Samples should be taken from untreated areas, and products with potential cross resistance with the target product objective of the tests.

- Samples should be taken in a way that represents the total area in a diagonal or in a "W".
- A site or field sample are composed of fragments of leaves taken from 25 to 30 plants representing the all variation of the field, depending on whether the information is being collected for routine monitoring or base line construction. Base lines that represent large areas should take more points that those that represent specific areas. The objective is to cover the full range of the population. Samples should be taken during the early hours of the morning, but after the dew has dried.
- In each plant, leaf fragments of the youngest leaf found with spots at stage 5 and 6 bearing mature pseudothecia should be collected (Figure 1).
- Collected leaf samples should be transported in paper bags (never in plastic bags) to avoid transpiration wetting and ascospore release.
- Bags should be tagged with the site, sample number, sampling date, farm, field and record data on BSD severity and fungicide use. Any other specific data that may help with drawing conclusions on population sensitivity shift can be added. During transport exposure of the paper bags to high temperatures or wetting should be avoided.

5.2. General procedure of sample preparation for sensitivity tests

- At laboratory, leaf samples are allowed to dry overnight on a bench at room conditions. After that, samples can be stored in paper bags on a bench under fresh conditions until a maximum period of two weeks before being used in sensibility tests.
- Fragments collected can be checked under a stereomicroscope (20 X) to select the sectors bearing fructification bodies (pseudothecia and spermogonia; Figure 2 a).
- When samples are collected in a long dry season or have been stored in the laboratory for some time, they can be incubated for 24-48 hours in a bag with wet filter paper to favor pseudothecia and ascospore maturation (see Figure 4). This process should be carried out with care because it could induce ascospore release and result in loss of the sample.
- If fungal outgrowth contaminations are present, fragments can be superficially disinfected for a minute in 2% sodium hypochlorite and quickly rinsed with tap water.

5.2.1. Induction of ascospore release

Leaf fragments bearing spots with fruiting bodies are cut in rectangular pieces of approximately 3 x 3 cm and then:

a) Five to six pieces of leaves with spots are individually stapled to 9 cm filter papers leaving the upper side (adaxial surface) exposed (as shown in Figure 3b adaxial surface usually have more pseudothecia than abaxial surface). Paper disks with the stapled spotted leaf fragments are submerged in tap water in a tray for 4-5 minutes and then placed in lids of Petri plates with water agar amended with the fungicide being studied. The upper surface of leaf fragments should be in direction of the agar. On the bottom of the Petri plates, the fragments positions are marked with a permanent marker to facilitate ascospore localization and assessing. Plates are placed upside down in a laboratory bench for an hour (see Figure 5) to allow ascospore release and deposition on fungicide-amended water agar. Thereafter, the filter papers are removed from the lids.

b) When a large number of samples have to be tested, stapling the fragments to filter papers can be time consuming and tedious. An alternative procedure is to put the leaf pieces in a water tray for five minutes to allow the leaf tissue to imbibe the water and place them immediately without stapling, upper side up, on filter papers previously placed on Petri lids that are placed upside down on a laboratory bench. Later the lids with the filter paper and the leaf are covered with the bottom of the correspondent Petri plates with water agar amended with fungicides. The rest of the procedure is the same as explained in (a).

Incubation. After ascospore release is completed, the plates with ascospores in media with fungicide are incubated for 48 hours at 27 ºC. Date and time of start and end of incubation are recorded.

5.2.2. Preparation of tests for sensitivity to fungicides

Fungicides used for the tests are usually:

- Benomyl as 50% a.i. WP.
- Carbendazim as 25% a.i. WP
- Methyl thiophanate as 70% a.i. WP
- Propiconazole, difenoconazole, tebuconazole, triadimenol, as 25% a.i. EC
- Azoxystrobin as 25% SC.
- Tridemorph and fenpropimorph as 45% a.i., E.C.
- Pyrimethanil as 60% EC.

5.2.3. Preparation of stock solutions

The amount of stock solution to be prepared will depend on the amount of samples to be analyzed. The solvent can be water, acetone or absolute alcohol, depending on the formulation of the active ingredient. The final solution and the amount of fungicides to be prepared can be calculated from the following procedure:

1. 50 mg a.i. + solvent to complete 100 ml of final solution = 0.5 mg/ml = 500 µg/ml = 500 ppm ⟶ **S1.**

To obtain 50 mg a.i. is required to measure:

- 62.5 mg or ml of a formulation at 80% a.i.
- 71.42 mg or ml of a formulation at 70% a.i.
- 83.3 mg or ml of a formulation at 60% a.i.
- 100.0 mg or ml of a formulation at 50% a.i.
- 111.1 mg or ml of a formulation at 45% a.i.
- 150.15 mg or ml of a formulation at 30% a.i.
- 200.0 mg or ml of a formulation at 25% a.i.

2. 5 ml of **S1** + solvent to complete 50 ml of final solution = 0.05 mg/ml = 50 µg/ml = 50 ppm ⟶ **S2**

3. 5 ml of **S2** + solvent to 50 ml of final solution
   = 0.005 mg/ml = 5 µg/ml = 5 ppm → **S3**
4. 1mL of **S3** + solvent to 50 ml of final solution
   = 0.1mg/ml = 0.1 µg/ml = 0.1 ppm → **S4**
5. 1 ml **S4** + solvent to 100 ml of final solution
   = 0.001 mg/ml = 0.001 µg/ml = 0.001 ppm → **S5**

Fungicides solutions should be added to agar when is fully melted and fresh enough to keep in hand. After adding the fungicide solution, the agar should be gently shaken to allow full homogenization of the fungicide in the agar, taking care to avoid bubble formation. Then the plates are kept in a laboratory bench at room temperature until hardening.

Dilutions to prepare agar amended with different concentrations of fungicides. **(Should be use high purity agar to be able to compare results).**		
Concentration (µg/ml)	**Amount of stock solution**	**Amount of water agar (ml)**
0	-	100
0.0001	1 ml of S5	99.0
0.001	1 ml of S4	99.0
0.01	5 ml of S3	95.0
0.03	3 ml of S5	97.0
0.1	2 ml of S3	98.0
0.3	0.6 ml of S2	99.4
0.5	1 ml of S2	99.0
1	2 ml S2	98.0
3	0.6 ml of S1	99.4
5	1 ml S1	99.0
10	2 ml S1	98.0

5.3. General considerations on assessments and interpretations of results

During assessment and interpretation of results, it is important consider the following:

- Assessment should be carried out only on *M. fijiensis* spores. As was previously explained, in black Sigatoka spots *M. minima* and *M. musae* can also be found (see Figures 6, 7 and 8 and descriptions). *M. fijiensis* ascospores have a length between 13 and 17 µm and germinate by two vigorous tubes that grow in the main axis of the spores.
- A spore is considered 'germinated' when tubes have >5 µm long.
- Different people can produce different results. Unlikely as it may seem, this can happen. Experience shows that it is best to have all work conducted by the same technician or scientist, or groups of the same, for as long as possible. They are most likely due to slight differences in interpretation of a protocol and experimental procedures. They can be

controlled by the use of standard reference isolates and, where possible, a period of joint working so that differences are identified and corrected.

- Baselines developed and published by other laboratories should not be used. The only way to prevent problems is to generate a baseline for a particular laboratory or to exchange reference isolates such that individual laboratories can use 'bridging data' to enable valid comparisons.

5.4. Monitoring procedure for different chemical families

5.4.1. Benzimidazole

Development of a reference base line

A base line should be defined as early as possible before the fungicide is widely used. In the case of benzimidazole, this is difficult because the fungicide has been in use for a long time. So, it is necessary to draw a base line monitoring, sampling as much different sites as possible and always using a *M. fijiensis* population of a relatively isolated and untreated location to obtain a "wild type or nearly" population that would be used systematically as reference.

To develop a base line, it is recommended that not less than thirty samples are collected from the field. The samples are selected, collected and transported and stored as previously described.

Monitoring

Concentrations used for monitoring are 0, 1 and 5 ppm (Anon. 1980; Pérez and Mauri, 1982; Smith *et al*., 1992).

Amended culture media preparation: This can be prepared from the stock solutions and dilutions described in the preparation of dilutions or also by the following procedure: Include in Erlenmeyer flasks the water agar required to obtain the benomyl concentrations of 0, 0.5, 1.0, 5.0, 10 ppm. Weigh 100 mg of benomyl (a.i.) and dilute in 100 ml sterile distilled water to obtain a stock solution with a 1000 ppm active ingredient. From the stock solution prepare the following concentrations of benomyl:

Concentration (ppm)	**ml of stock sol.**	**ml of water agar.**
0	0	200.0
0.5	0.1	199.9
1	0.2	199.8
5	1	199.0
10	2	198.0

Benomyl in water solutions are added to water agar after sterilization when media is still fully liquid but at around 50-55ºC. The flasks are then gently moved for homogenization, taking care to prevent development of bubbles. Then the amended agar is dispensed in three to four Petri plates (replications) for concentration and allowed to solidify at room temperature.

When agar is cold and hard, allow samples to discharge ascospores and incubate for 48 hours following the procedure previously indicated. It is very important to record time of beginning and ending incubation at 27 ºC.

After 48 hours of incubation, the plates are placed in an incubator or refrigerator at temperatures below 10ºC to stop any further germination or growth. Plates are removed of incubator only at the moment of incubation.

Assessment

The assessment is qualitative based on normal, abnormal or non-germinated spores. The Minimal inhibitory Concentration (MIC) is 1ppm. Spores with normal germination at 1 ppm and 5 ppm (RF= or >5) are consider resistant. Values of inhibition for each concentration are calculated with the formula: Inhibition at concentration n (%) = [(a-b)/a]*100 where:

a) germination in the control;

b) germination at concentration n;

After determining that a population is resistant, a second test should be carried out to determine ED_{50} and ED_{99} using larger set of concentrations. For this, a set of at least 5 concentrations (more would be better) should be included to obtain a good regression and estimation of ED_{50} and ED_{99} values (see "ED50 and ED99 calculation"). It is important to compare the results of a treated with an untreated reference population or field. In Annex 2, recording forms for benzimidazole tests are provided.

5.4.2. SBI's (DMI's and amines)

As has been previously explained, sensitivity tests for SBI's are quantitative and based on the growth of the ascospore germ tubes in a set of different fungicide concentrations that cover all possible range of sensitivities that can be found in populations. For this group of fungicides, a discriminatory threshold concentration for all member of the group cannot be used, because: a) a single value can measure different parts of the population for each active ingredient member of the group; b) results are statistically questionable; c) if the concentration used is very low, fluctuations could occur and if are very high the test can have a low sensitivity.

The tests are carried out with the following concentrations of active ingredients:

5.4.2.1. *DMI's* (propiconazole, hexaconazole, tebuconazole, triadimenol, flusilazole, bromuconazole, cyproconazole, epoxyconazole, etc.)

- *For base line determination:* 0, 0.0001, 0.001, 0.01, 0.1, 1.0 μg/ml. (in areas with a large history of DMI's use the concentration of 0.0001 can be unnecessary due to sensitivity shift).
- *For monitoring:* 0, 0.001, 0.01, 0.1, 1 μg/ml

5.4.2.2. *Amines* (tridemorph, fenpropimorph and spiroxamine):

- For base line determination: 0, 0.001, 0.01, 0.1, 1 and 10 μg/ml.
- *For monitoring:* 0, 0. 01, 0.1, 1, 10 μg/ml

Agar poisoning with fungicides, agar dispensing in plates, ascospore discharge and incubation procedures are as already explained.

In the case of SBI's fungicides, it is of paramount importance to have a careful control of the period of growth in incubation for 48 hours because the quantitative assessment based on length of germ tube is laborious and different times of assessment could give a significant difference for different concentrations. So, to avoid variations due to different incubation periods, it is mandatory to stop the growth of germ tube at end of 48 hours of incubation,

placing the plates at 5-10 °C in a refrigerator and remove from it only at the moment of start assessing.

The length of germ tubes are measured under a microscope with a ocular micrometer at 40 X magnification. With this data the following is determined:

- *DE $_{50}$ and DE $_{99}$.* (See the different procedures).
- *Frequency distribution of germ tube inhibition at concentrations of 0.001, 0.01 and 0.1 µg/ml of active ingredient***.** For this, the mean of the length of ascospores germ tubes in the control is calculated and the limit values corresponding to 10%, 30%, 50% and 70 % of inhibition. Then, the number of spores that are in the inhibition ranges of 0-10 %, 10.1-30%, 30.1-50%, 50.1-70% and >70% is determined for each concentration. With this data a graph of frequency distribution is plotted as shown in Figure 23.

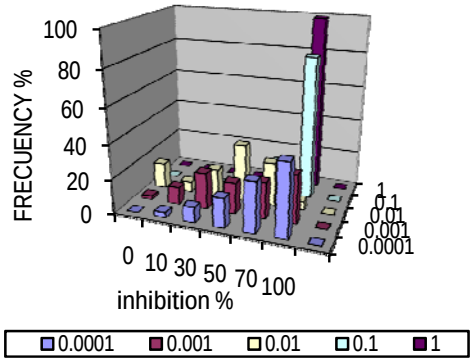


Figure 23 . Example of a frequency distribution graph of ascospores germ tube inhibition at different concentrations of propiconazole

- *Frequency distribution of DE$_{50}$ values determined for populations of a locality or a site.* DE$_{50}$ data is classified in nine ranges or classes: 1= 0.0001-0.0003; 2= 0.0003-0.001; 3= 0.001-0.003; 4= 0.003-0.01; 5= 0.01-0.03; 6= 0.03- 0.1; 7= 0.1-0.3; 8= 0.3 - 1.0; 9=>1.0 (µg/ml). With the DE$_{50}$ values data of a site or from a number of farms or fields in a location, a frequency distribution graph over time can be constructed, that illustrates the shift of sensitivity of populations as shown in Figure 24 with data of Cuba (Pérez *et al.,* 2006).

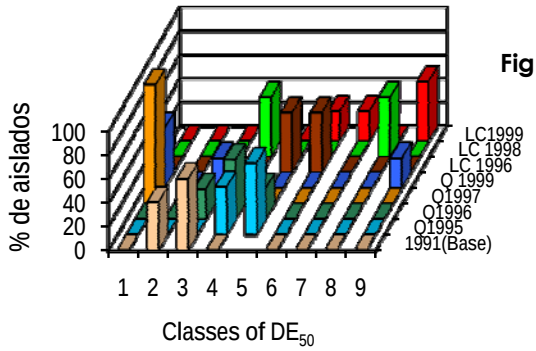


Figure 24. Example of distribution frequency of ED$_{50}$ of *M. fijiensis* populations to propiconazole in different years on La Cuba banana enterprise in Cuba. In the example is shown a shift of sensibility to propiconazole in time Pérez *et al*, (2003).

5.4.3. QoI's (azoxystrobin, trifloxystrobin, pyraclostrobin)

Resistance to QoI's are mediated by single gene mutation leading to changes in the target site. Selection process is disruptive. Monitoring can be carried out using MIC of 1 ppm.

- *For base line determination:* The test is carried out by measuring germination and germ tube growth at 0, 0.1, 0.5, 1, and 5 µg a.i./ml concentrations. In some cases it is favorable to include a 10 ppm concentration for get a better fitness of curves. Germination and germ tube growth of sensitive isolates are totally inhibited at 1µg/ml. It is recommended for some specialist to consider germinated ascospores with a tube > 150 µ of length.
- *Monitoring:* The test can be carried out at concentrations of 0, 1 and 5 µg a.i./ml. Germination at concentrations of 5 µg a.i./ml with tubes of more 150 µm are a clear indication of resistance.

Figure 25 demonstrates the curves of *M. fijiensis* ascospore germination at different concentrations of QoI's fungicides (trifloxystrobin resistant and sensitive *M. fijiensis* populations of Costa Rica (Chin et al, 2001); azoxystrobin-sensible populations of Cuba (Pérez *et al.*, 2004).

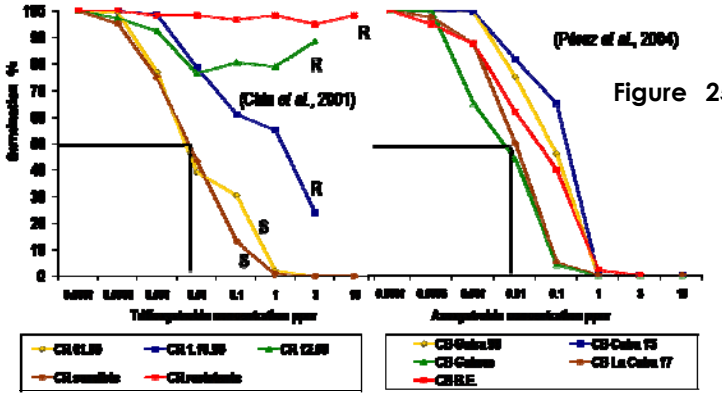


Figure 25. Effect of trifloxystrobin and azoxystrobin on inhibition of ascospore germination of sensitive and resistant Costa Rica (Chin et al., 2001) and Cuba (Pérez *et al.*, 2004) *M. fijiensis* populations respectively.

Sensitive populations in both cases are inhibited at 1 ppm while resistant populations of Costa Rica (R), show high levels of germination at 3, 5 and 10 µg a.i./ml.

5.4.4. Anylinopyrimidines: Pyrimethanil

Pyrimethanil inhibit protein biosynthesis, causing dehydration and collapse of ascospore germ tubes inhibiting further growth. A broad distribution in sensitivity to pyrimethanil has been observed and ED50 values varied from 3 to 80 µg /ml and from 5 to 80 µg /ml respectively (Duvert et al., 2002). Available base lines for pyrimethanil are from Costa Rica. So far sensitivity to pyrimethanil has been stable in most banana production areas (FRAC, 2012).

Base line determination and monitoring: Base line and monitoring are carried out using the same concentrations of pyrimethanil. Base line should be developed with a large number of samples. For this, the following concentrations are employed: 0, 1, 10, 30 and 100 µg a.i./ml, with at least two replications of each concentration.

6. Calculation of effect dosage 50 (ED_{50}) and 99 (ED_{99})

ED_{50} and ED_{99} are defined as dosages that cause a 50 and 99 % of growth inhibition respectively (also inhibitory concentrations 50 and 99).

The curves of mortality or inhibition over fungicide concentration are sigmoid. To precisely estimate the values of inhibition to each concentration it is necessary to adjust the curve to a straight line. The general procedure is:

1. Percentage inhibition values are transformed to probit units (unit of measure of statistical probability based on deviation of normal distribution). A probit values table is provided at Annex 2.
2. Concentrations are transformed to log of concentration.
3. The regression equation of probit values to log concentration (slope, regression coefficients and significance) is determined. Excel and several statistical packages can be used for the regression analysis.
4. From the straight line, the log concentration that inhibit 50 (probit value 5) and 99% (probit value 7.32) of growth compared to the untreated control are determined.
5. Antilog of concentrations to determine ED_{50} and ED_{99}.

Different procedures are available to determine the ED_{50} and ED_{99}.

6.1. Calculation of ED50 using logarithmic paper

In the following example (Figure 25) the data of Chin *et al. (*2001) was used to calculate DE_{50} and DE_{99} and resistance factor (RF) of trifloxystrobin (QoI) sensitive and resistant *M. fijiensis* isolates using logarithmic paper. In the example, DE_{99} of the resistant population was not estimated because it was outside the range of concentrations tested in the analysis.

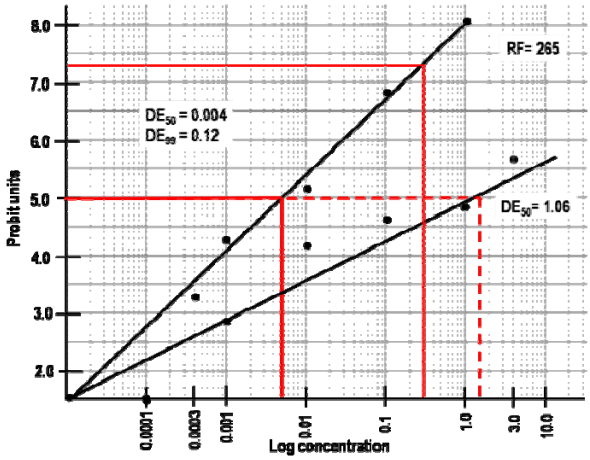


Figure 25. Determination of DE_{50}, DE_{99} and resistance factor (RF) of *M. fijiensis* resistant and susceptible isolates to trifloxystrobin (QoI). Data adapted from Chin *et al.*, (2001).

6.2. *Determination using statistical software*

Different software and procedures can be used to calculate DE_{50} and DE_{99}.

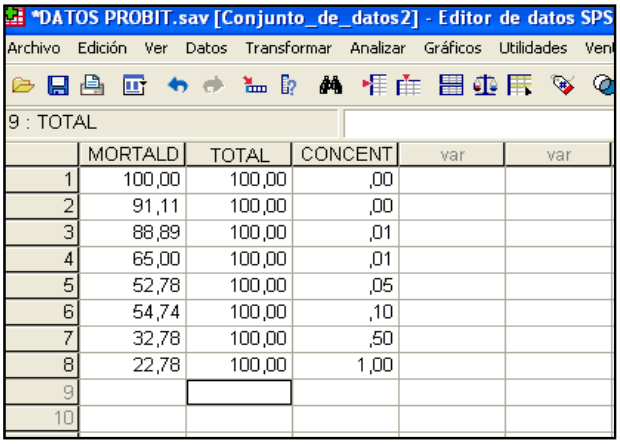
ESTIMATION OF DE_{50} AND DE_{99} USING THE STATISTICAL PACKAGE SPSS 15

Data should be order in a matrix in the following way:

CONCENTRATION	REPLICATION 1	REPLICATION 2	REPLICATION Y	MEAN
C_1	$INHIBITION_{1,1}$	$INHIBITION_{1,2}$	$INHIBITION_{1,y}$	MEAN (C_1)
C_2	$INHIBITION_{2,1}$	$INHIBITION_{2,2}$	$INHIBITION_{2,y}$	MEAN (C_2)
C_3	$INHIBITION_{3,1}$	$INHIBITION_{3,2}$	$INHIBITION_{3,y}$	MEAN (C_3)
C_N	$INHIBITION_{N,1}$	$INHIBITION_{N,2}$	$INHIBITION_{N,y}$	MEAN (C_N)

Where: C_3, is the third level of concentration used and $Inhibition_{3,2}$ is the percentage of inhibition in the 2nd replication of the 3rd concentration and MEAN C_3 is the arithmetic mean of all inhibition percentages of concentration C3.

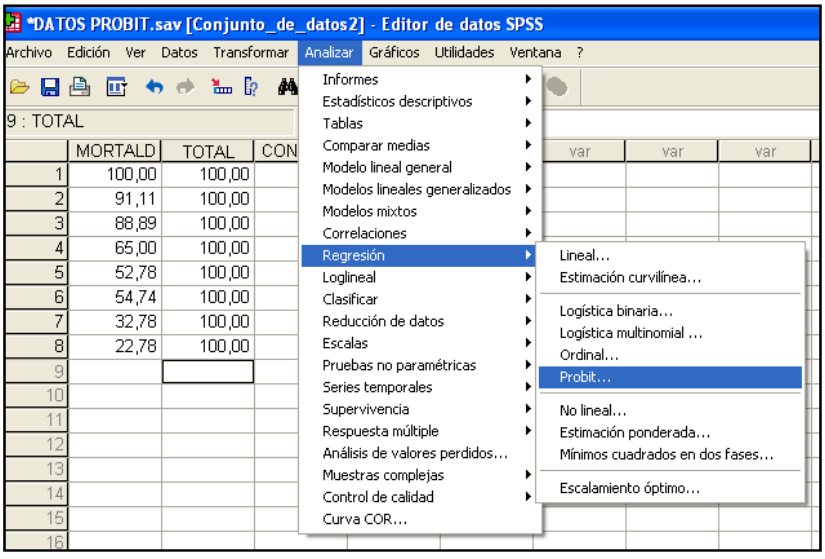
Data organized in the matrix is then input in the statistical package SPSS 15 organized in three columns as follows:

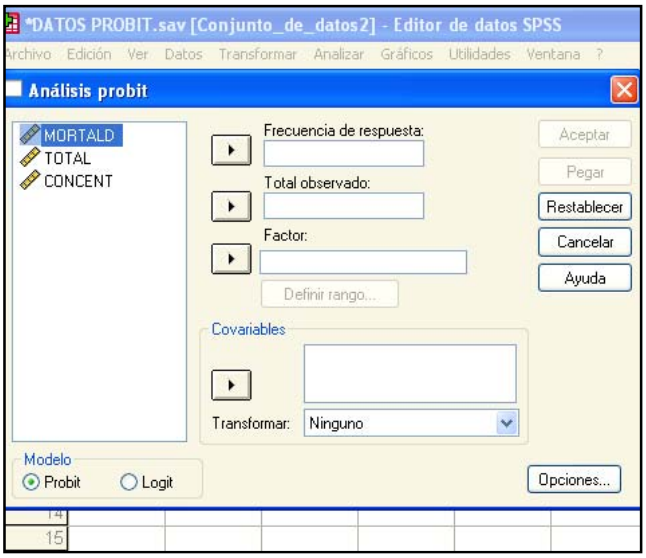


In the first column "**MORTALD**" the means of the values of % of mortality (inhibition) are entered, the eight concentration values used in the column "**CONCENT**". In the 2nd column "**TOTAL**" the total value of mortality estimation (100%) is expressed. Note there is an inverse relation between concentration and inhibition or mortality.

After data is entered, the Probit Regression analysis is carried out.

For this, click in the upper bar of the display in "**Analizar**" (Analyze)/ "**Regresión**" (Regression)/ Probit. Then the following window of dialog appears:



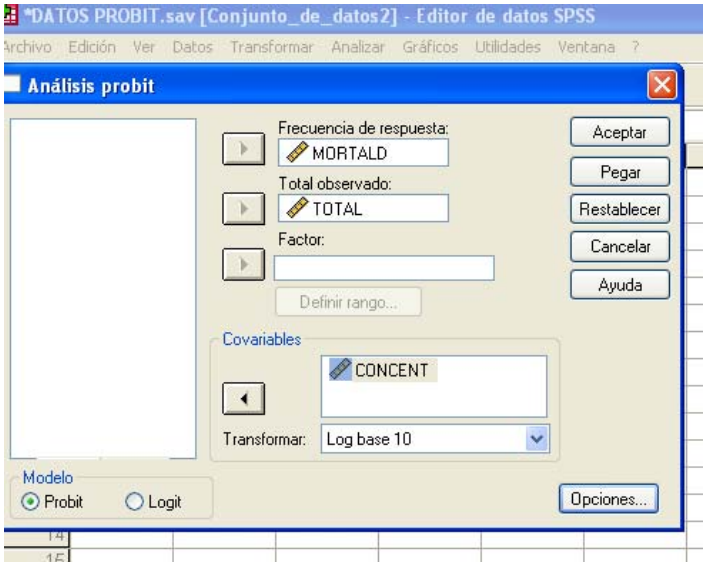


Data requested in the left part are introduced in the blanks as follow:

"MORTALD" (inhibition)►Frecuencia de respuesta (Mean of percentage inhibition)

"TOTAL" ►Total observed (100)

"CONCENT" (Concentration)► Covariable (log base 10). For this, open the window "Transformar" (to transform) and select Log base 10, that will allow transform concentration values to log of concentrations. This window will show up as follows:



Click in **"Aceptar"**(accept) and results of the probit regression will appear

One of the output tables is the Regression of Probit-Concentration, shown below.

Parameters estimation

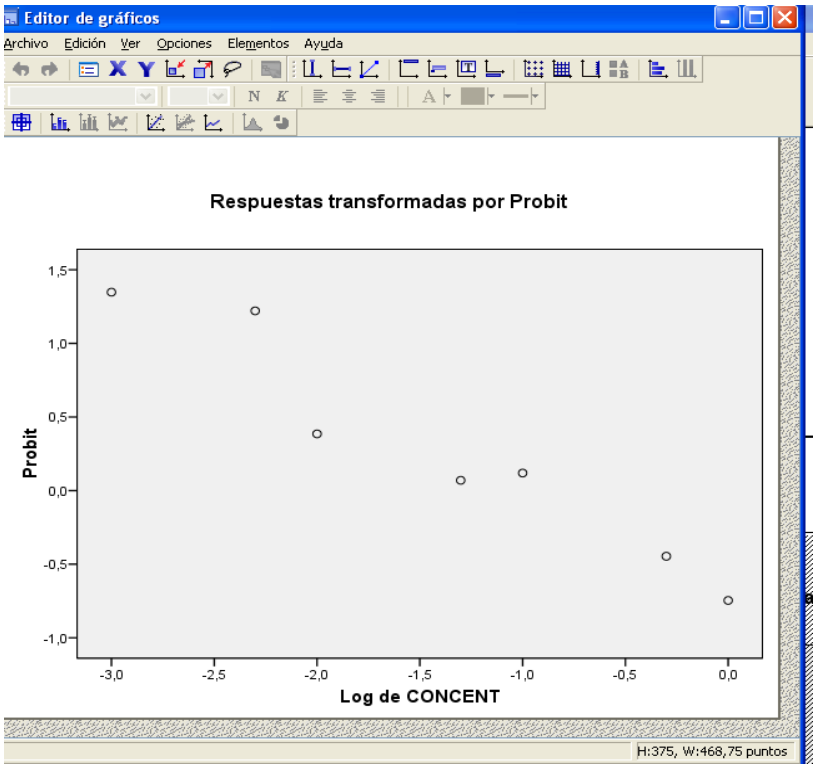
	Parameter	Estimate	Standard error	Z	Sig.	Confidence interval at 95%	
		Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit
PROBIT (a)	CONCENT	-,684	,056	-12,203	,000	-,794	-,574
	Intersection	-,703	,089	-7,938	,000	-,792	-,615

(a) PROBIT Model: PROBIT (p) = Intersection + BX (covariable X are transformed using logarithm of base 10)

The equation then will be: **PROBIT** = - 0.703 – 0.684*LOG (CONCENT)

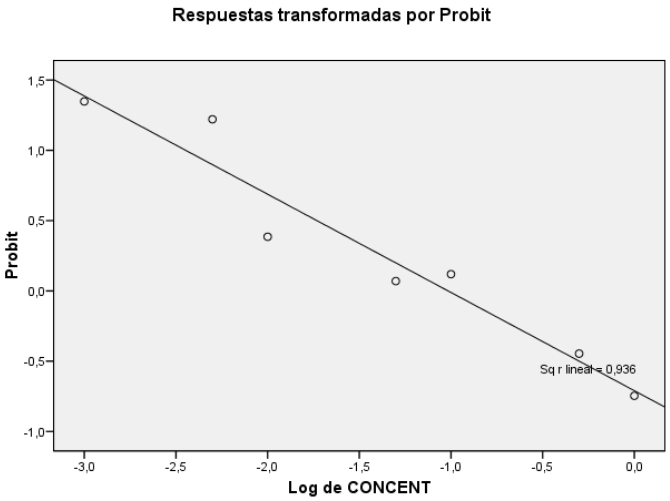
From table can be concluded from the level of signification (0.000 for intercept and slope) that both are distinct of zero, which means that equation values are valid.

The graph showing the regression between both variables is shown at end of the results and can be edited to visualize lineal regression adjustment and to estimate the determination coefficient. For this, double click on the graph and editor will show up as follows:



In the 3rd bar row where the button that allows drawing different adjustment lines appears, select the one 'total adjust' (5th button from left to right) and the adjusted regression line will be displayed. Close editor and the results graph with regression line and the correspondent R^2 coefficient will appear.

In this case, a good level of adjustment (R^2 =0.936) can be confirmed.



Finally, results are shown in a table with information of interest for the regression obtained:

Limits of confidence

	Probability	Confidence limits at 95% for CONCENT			Confidence limits at 95% for log CONCENT (a)		
		Estimation	Lower limit	Upper limit	Estimation	Lower limit	Upper limit
PROBIT (b)	,010	236,326	28,247	13448,559	2,374	1,451	4,129
	,020	94,384	13,950	3524,171	1,975	1,145	3,547
	,030	52,722	8,904	1508,715	1,722	,950	3,179
	,040	34,020	6,347	797,641	1,532	,803	2,902
	,050	23,822	4,816	475,265	1,377	,683	2,677
	,060	17,590	3,805	306,033	1,245	,580	2,486
	,070	13,482	3,094	208,140	1,130	,491	2,318
	,080	10,626	2,569	147,448	1,026	,410	2,169
	,090	8,557	2,169	107,807	,932	,336	2,033
	,100	7,010	1,856	80,840	,846	,268	1,908
	,150	3,071	,967	24,661	,487	-,014	1,392
	,200	1,594	,572	9,670	,202	-,242	,985
	,250	,908	,362	4,366	-,042	-,442	,640
	,300	,548	,238	2,157	-,261	-,624	,334
	,350	,343	,159	1,135	-,465	-,799	,055
	,400	,220	,107	,626	-,658	-,970	-,203
	,450	,143	,072	,358	-,844	-1,144	-,446
	,500	**,094**	**,047**	**,212**	-1,028	-1,324	-,674
	,550	,061	,030	,129	-1,212	-1,517	-,891
	,600	,040	,019	,080	-1,399	-1,727	-1,097
	,650	,026	,011	,051	-1,592	-1,958	-1,296

	Probability	Confidence limits at 95% for CONCENT			Confidence limits at 95% for log CONCENT (a)		
		Estimation	Lower limit	Upper limit	Estimation	Lower limit	Upper limit
	,700	,016	,006	,032	-1,795	-2,215	-1,492
	,750	,010	,003	,020	-2,015	-2,505	-1,691
	,800	,006	,001	,013	-2,259	-2,838	-1,903
	,850	,003	,001	,007	-2,544	-3,235	-2,140
	,900	,001	,000	,004	-2,902	-3,743	-2,431
	,910	,001	,000	,003	-2,989	-3,867	-2,500
	,920	,001	,000	,003	-3,083	-4,001	-2,575
	,930	,001	,000	,002	-3,186	-4,150	-2,656
	,940	,000	,000	,002	-3,302	-4,316	-2,748
	,950	,000	,000	,001	-3,433	-4,506	-2,851
	,960	,000	,000	,001	-3,588	-4,730	-2,972
	,970	,000	,000	,001	-3,778	-5,005	-3,120
	,980	,000	,000	,000	-4,031	-5,372	-3,317
	,990	,000	,000	,000	-4,430	-5,952	-3,625

a) Logarithm base 10.; b) A heterogenic factor is used.

In this table, the two first columns are of interest, where results of the regression of concentration values and inhibition probability are already transformed. For a 50% of inhibition or mortality (probability of 0.50) the concentration level is 0.094. It can be also interpreted inversely; if you want to determine the concentration for 60% of survival, this will work out to be 0.22. You can estimate the concentration that causes a given level of mortality or inhibition. For instance, concentration causing 20 % of inhibition is 1.594 µg/ml.

Columns 2, 3 and 4 are confidence intervals for 95% of probability. At an inhibition level of 50%, the concentration interval is between 0.047 and 0.212.

Any other values that do not appear in the table, have to be estimated from the regression equation determined, after performing appropriate transformations to log of concentration and probit of inhibition or mortality.

Finally it must be emphasized that, equations are valid only in the range of concentrations used in the tests and should not be used to estimate other values out of this range.

7. Annexes

ANNEX 1. Equipment, materials and reagents

Equipment:

1. Optical microscope	1
2. Stereoscope	1
3. Autoclave	1
4. Incubator	1
5. Distiller	1
6. Refrigerator	1
7. Computer	1
8. Boxer or sterile air flow bench	1

Materials

1. Stapler	1
2. Staples (box)	1
3. Dissection needles	1
4. Microbiological needles	1
5. Scissors	1
6. Forceps fine point	1
7. Petri plates 5-8 cm.	50
8. Petri plates 10 cm	100
9. Petri plates of 20 cm	50
10. Culture tubes	200
11. Micropipette automatic 1-1000µl	1
12. Micropipette automatic 1-200µl	1
13. Yellow tips	400
14. Blue tips	400
15. Aspirator auxiliary for glass macro-pipettes	1
16. Graduated pipettes of 1ml	10
17. Graduated pipettes of 5 ml	10
18. Graduated pipettes of 10 ml	10
19. Graduated glass cylinder 50 and 100 ml	2 each
20. Graduated glass cylinder 1L	1
21. Glass slides for microscopy boxes	2
22. Cover slips (box)	2
23. Filter paper Whatman No. 1 10 cm (box)	5

24. Permanent markers (fine) 2
25. Logarithm paper (sheets) 50
26. Paper bags large for samples 200
27. Paper bags small for samples 400

Reagents, media and chemicals

1. Agar No 3. Oxoid 1kg
2. Potato dextrose agar 1kg
3. Dextrose 1kg
4. Calcium carbonate reactive 500 g
5. V-8 Juice flasks or cans of 250 ml 10
6. Benomyl 50% a.i. WP.
7. Carbendazim 25% a.i. WP
8. Methyl thiophanate 70% a.i. WP
9. Propiconazole, difenoconazole, tebuconazole, triadimenol, 25% a.i. EC
10. Azoxystrobin 25% SC.
11. Tridemorph and fenpropimorph 45% a.i., E.C.
12. Pyrimethanil 60% EC.

ANNEX 2. Conversion Table: inhibition values to Probit units (adapted from Bliss, 1937)

Inhibition values transformed to probit units										
Units	Decimals									
	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0		1.9098	2.1218	2.2522	2.3479	2.4242	2.4879	2.5427	2.5911	2.6344
1	2.6737	2.7096	2.7429	2.7738	2.8027	2.8299	2.8556	2.8799	2.9031	2.9251
2	2.9463	2.9665	2.9859	3.0046	3.0226	3.0400	3.0369	3.0732	3.0890	3.1043
3	3.1192	3.1337	3.1478	3.1616	3.1750	3.1881	3.2009	3.2134	3.2256	3.2376
4	3.2493	3.2608	3.2721	3.2831	3.2940	3.3046	3.3151	3.3253	3.3354	3.3454
5	3.3551	3.3648	3.3742	3.3836	3.3928	3.4018	3.4107	3.4195	3.4282	3.4308
6	3.4452	3.4536	3.4618	3.4699	3.4780	3.4859	3.4937	3.5015	3.5091	3.5167
7	3.5242	3.5316	3.5389	3.5162	3.5534	3.5603	3.5675	3.5745	3.5813	3.5882
8	3.5949	3.6016	3.6083	3.6148	3.6213	3.6278	3.6342	3.6405	3.6468	3.6531
9	3.6592	3.6654	3.6715	3.6775	3.6835	3.6894	3.6953	3.7012	3.7070	3.7127
10	3.7184	3.7241	3.7298	3.7354	3.7409	3.7464	3.7519	3.7574	3.7628	3.7681
11	3.7735	3.7788	3.7840	3.7893	3.7945	3.7996	3.8048	3.8099	3.8150	3.8200
12	3.8250	3.8300	3.8350	3.8399	3.8448	3.8497	3.8545	3.8593	3.8641	3.8689
13	3.8736	3.8783	3.8830	3.8877	3.8923	3.8969	3.9015	3.9061	3.9107	3.9152
14	3.9197	3.9242	3.9286	3.9331	3.9375	3.9419	3.9463	3.9506	3.9550	3.9593
15	3.9636	3.9678	3.9721	3.9763	3.9806	3.9848	3.9890	3.9931	3.9973	4.0014
16	4.0055	4.0096	4.0137	4.0178	4.0218	4.0259	4.0299	4.0339	4.0379	4.0419
17	4.0458	4.0498	4.0537	4.0576	4.0615	4.0654	4.0693	4.0731	4.0770	4.0808
18	4.0846	4.0884	4.0922	4.0960	4.0998	4.1035	4.1073	4.1110	4.1147	4.1184
19	4.1221	4.1258	4.1295	4.1331	4.1367	4.1404	4.1440	4.1476	4.1512	4.1548
20	4.1584	4.1619	4.1655	4.1690	4.1726	4.1761	4.1796	4.1831	4.1866	4.1901
21	4.1936	4.1970	4.2005	4.2039	4.2074	4.2108	4.2142	4.2176	4.2210	4.2244
22	4.2278	4.2312	4.2345	4.2379	4.2412	4.2446	4.2479	4.2512	4.2546	4.2579
23	4.2612	4.2644	4.2677	4.2710	4.2743	4.2775	4.2808	4.2840	4.2872	4.2905
24	4.2937	4.2969	4.3001	4.3033	4.3065	4.3097	4.3129	4.3160	4.3192	4.3224
25	4.3255	4.3287	4.3318	4.3349	4.3380	4.3412	4.3443	4.3474	4.3505	4.3536
26	4.3567	4.3597	4.3628	4.3659	4.3689	4.3720	4.3750	4.3781	4.3811	4.3842
27	4.3872	4.3902	4.3922	4.3962	4.3992	4.4022	4.4052	4.4082	4.4112	4.4142
28	4.4172	4.4201	4.4231	4.4260	4.4290	4.4319	4.4349	4.4378	4.4408	4.4437
29	4.4466	4.4495	4.4524	4.4554	4.4583	4.4612	4.4641	4.4670	4.4698	4.4727
30	4.4756	4.4785	4.4813	4.4842	4.4871	4.4899	4.4928	4.4956	4.4985	4.5013
31	4.5041	4.5070	4.5098	4.5126	4.5155	4.5183	4.5211	4.5239	4.5267	4.5295
32	4.5323	4.5351	4.5379	4.5407	4.5435	4.5462	4.5490	4.5518	4.5546	4.5573
33	4.5601	4.5628	4.5636	4.5684	4.5711	4.5739	4.5766	4.5793	4.5821	4.5848
34	4.5875	4.5903	4.5930	4.5957	4.5984	4.6011	4.6039	4.6066	4.6093	4.6120
35	4.6147	4.6174	4.6201	4.6228	4.6255	4.6281	4.6308	4.6335	4.6362	4.6389
36	4.6415	4.6442	4.6469	4.6495	4.6522	4.6549	4.6575	4.6602	4.6628	4.6655
37	4.6681	4.6708	4.6734	4.6761	4.6787	4.6814	4.6840	4.6866	4.6893	4.6915
38	4.6945	4.6971	4.6998	4.7024	4.7050	4.7076	4.7102	4.7129	4.7155	4.7181
39	4.7207	4.7233	4.7259	4.7285	4.7311	4.7337	4.7363	4.7389	4.7415	4.7441
40	4.7467	4.7492	4.7518	4.7544	4.7570	4.7596	4.7622	4.7647	4.7673	4.7699
41	4.7725	4.7750	4.7776	4.7802	4.7827	4.7853	4.7879	4.7904	4.7930	4.7955
42	4.7981	4.8007	4.8032	4.8058	4.8083	4.8109	4.8134	4.8160	4.8185	4.8211

Inhibition values transformed to probit units										
Units	Decimals									
	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
43	4.8236	4.8262	4.8287	4.8313	4.8338	4.8363	4.8389	4.8414	4.8440	4.8465
44	4.8490	4.8516	4.8541	4.8566	4.8592	4.8617	4.8642	4.8668	4.8693	4.8718
45	4.8743	4.8769	4.8794	4,8819	4,8844	4,8870	4,8895	4,8920	4,8945	4,8970
46	4.8996	4.9021	4.9046	4,9071	4,9096	4,9122	4,9147	4,9172	4,9197	4,9222
47	4.9247	4.9272	4.9298	4,9323	4,9348	4,9373	4,9398	4,9423	4,9448	4,9473
48	4.9498	4.9524	4.9549	4,9574	4,9599	4,9624	4,9649	4,9674	4,9699	4,9724
49	4.9749	4.9774	4.9799	4,9825	4,9850	4,9875	4,9900	4,9925	4,9950	4,9975
50	5,0000	5,0025	5,0050	5.0075	5,0100	5,0125	5,0150	5,0175	5,0201	5.0226
51	5.0251	5,0276	5,0301	5.0326	5.0351	5,0376	5,0401	5,0426	5,0451	5.0476
52	5.0502	5,0527	5,0552	5.0577	5.0602	5,0627	5,0652	5,0677	5,0702	5.0728
53	5.0753	5,0778	5,0803	5.0828	5.0853	5,0878	5,0904	5,0929	5,0954	5.0979
54	5.1004	5,1030	5,1055	5,1080	5.1105	5,1130	5,1156	5,1181	5,1206	5.1231
55	5,1257	5,1282	5,1307	5,1332	5,1358	5,1383	5.1408	5,1434	5,1459	5,1484
56	5,1510	5,1535	5,1560	5,1586	5,1611	5,1637	5.1662	5,1687	5,1713	5,1738
57	5,1764	5,1789	5,1815	5,1840	5,1866	5,1891	5.1917	5,1942	5,1968	5,1993
58	5,2019	5,2045	5,2070	5,2096	5,2121	5,2147	5.2173	5,2198	5,2224	5,2250
59	5,2275	5,2301	5,2327	5,2353	5,2378	5,2404	5,2430	5,2456	5,2482	5,2508
60	5.2533	5,2559	5,2585	5,2611	5,2637	5,2663	5,2689	5,2715	5,2741	5,2767
61	5.2793	5,2819	5,2845	5,2871	5,2898	5,2924	5,2950	5,2976	5,3002	5,3029
62	5.3055	5,3081	5,3107	5,3134	5,3160	5,3186	5,3213	5,3239	5,3266	5,3292
63	5.3319	5,3345	5,3372	5,3398	5,3425	5,3451	5,3478	5,3505	5,3531	5,3558
64	5.3585	5,3611	5,3638	5,3665	5,3692	5,3719	5,3745	5,3772	5,3799	5,3826
65	5.3853	5,3880	5,3907	5,3934	5,3961	5,3989	5,4016	5,4043	5,4070	5,4097
66	5.4125	5,4152	5,4179	5,4207	5,4234	5,4261	5,4289	5,4316	5,4344	5,4372
67	5.4399	5,4427	5,4454	5,4482	5,4510	5,4538	5,4565	5,4593	5,4621	5,4649
68	5.4677	5,4705	5,4733	5,4761	5,4789	5,4817	5,4845	5,4874	5,4902	5,4930
69	5.4959	5,4987	5,5015	5,5044	5,5072	5,5101	5,5129	5,5158	5,5187	5,5215
70	5.5244	5,5273	5,5302	5,5330	5,5359	5,5388	5,5417	5,5446	5,5476	5,5505
71	5.5534	5,5563	5,5592	5,5622	5,5631	5,5681	5,5710	5,5740	5,5769	5,5799
72	5.5828	5,5858	5,5898	5,5918	5,5948	5,5978	5,6008	5,6038	5,6068	5,6098
73	5.6128	5,6158	5,6189	5,6219	5,6250	5,6280	5,6311	5,6341	5,6372	5,6403
74	5.6433	5,6464	5,6495	5,6526	5,6557	5,6588	5,6620	5,6651	5,6682	5,6713
75	5,6745	5,6776	5,6808	5,6840	5,6871	5,6903	5,6935	5,6967	5,6999	5,7031
76	5,7063	5,7095	5,7128	5,7160	5,7192	5,7225	5,7257	5,7290	5,7323	5,7356
77	5,7388	5,7421	5,7454	5,7488	5,7521	5,7554	5,7588	5,7621	5,7655	5,7688
78	5,7722	5,7756	5,7790	5,7824	5,7858	5,7892	5,7926	5,7961	5,7995	5,8030
79	5,8064	5,8099	5,8134	5,8169	5,8204	5,8239	5,8274	5,8310	5,8345	5,8381
80	5.8416	5,8452	5,8488	5,8524	5,8560	5,8596	5,8633	5,8669	5,8705	5,8742
81	5.8779	5,8816	5,8853	5,8890	5,8927	5,8965	5,9002	5,9040	5,9078	5,9116
82	5.9154	5,9192	5,9230	5,9269	5,9307	5,9346	5,9385	5,9424	5,9463	5,9502
83	5.9542	5,9581	5,9621	5,9661	5,9701	5,9741	5,9782	5,9822	5,9863	5,9904
84	5.9945	5,9986	6,0027	6,0069	6,0110	6,0152	6,0194	6,0237	6,0279	6,0322
85	6.0364	6,0407	6,0450	6,0494	6,0537	6,0581	6,0625	6,0669	6,0714	6,0758
86	6.0803	6,0848	6,0893	6,0939	6,0985	6,1031	6,1077	6,1123	6,1170	6,1217
87	6.1264	6,1311	6,1359	6,1407	6,1455	6,1503	6,1552	6,1601	6,1650	6,1700

Inhibition values transformed to probit units										
Units	Decimals									
	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
88	6.175	6,1800	6,1850	6,1901	6,1952	6,2004	6,2055	6,2107	6,2160	6,2212
89	6.2265	6,2319	6,2372	6,2426	6,2481	6,2536	6,2591	6,2646	6,2702	6,2759
90	6,2816	6,2873	6,2930	6,2988	6,3047	6,3106	6,3165	6,3225	6,3285	6,3346
91	6,3408	6,3469	6,3532	6,3595	6,3658	6,3722	6,3787	6,3852	6,3917	6,3984
92	6,4031	6,4118	6,4187	6,4255	6,4325	6,4395	6,4466	6,4538	6,4611	6,4684
93	6,4758	6,4833	6,4909	6,4985	6,5063	6,5141	6,5220	6,5301	6,5382	6,5464
94	6,5548	6,5632	6,5718	6,5805	6,5893	6,5982	6,6072	6,6164	6,6258	6,6352
95	6.6449	6.6546	6.6646	6,0747	6,6849	6,6954	6,7060	6,7169	6,7279	6,7392
96	6.7507	6.7624	6.7744	6,7866	6,7991	6,8119	6,8250	6,8384	6,8522	6,8663
97	6.8808	6.8957	6,9110	6,9268	6,9431	6,9600	6,9774	6,9954	7,0141	7,0335

Units	Decimals									
	0,00	0,01	0,02	0,03	0,04	0.05	0,06	0,07	0,08	0,09
98,0	7.0537	7,0558	7,0579	7,0600	7,0621	7,0642	7,0663	7,0684	7,0706	7.0727
98,1	7.0749	7,0770	7,0792	7,0814	7,0836	7,0858	7,0880	7,0902	7,0924	7.0947
98,2	7.0969	7,0992	7,1015	7,1038	7,1060	7,1084	7,1107	7,1130	7,1154	7.1177
98,3	7.1201	7,1224	7,1248	7,1272	7,1297	7,1321	7,1345	7,1370	7,1394	7.1419
98,4	7.1444	7,1469	7,1494	7,1520	7,1545	7,1571	7,1596	7,1622	7,1648	7.1675
98.5	7.1701	7,1727	7,1754	7,1781	7,1808	7,1835	7,1862	7,1890	7,1917	7,1945
98.6	7.1973	7,2001	7,2029	7,2058	7,2086	7,2115	7,2144	7,2173	7,2203	7,2232
98.7	7.2262	7,2292	7,2322	7,2353	7,2383	7,2414	7,2445	7,2476	7,2508	7,2539
98.8	7.2571	7,2603	7,2636	7,2668	7,2701	7,2734	7,2768	7,2801	7,2835	7,2869
98.9	7.2904	7,2938	7,2973	7,3009	7,3044	7,3080	7,3116	7,3152	7,3189	7,3226
99,0	7.3263	7,3301	7,3339	7,3378	7,3416	7,3455	7.3495	7,3535	7,3575	7,3615
99,1	7.3656	7,3698	7,3739	7,3781	7,3824	7,3867	7.3911	7,3954	7,3999	7,4014
99,2	7.4089	7,4135	7,4181	7,4228	7,4276	7,4324	7.4372	7,4422	7,4471	7,4522
99,3	7.4573	7,4624	7,4677	7,4730	7,4783	7,4838	7.4893	7,4949	7,5005	7,5063
99,4	7.5121	7,5181	7,5241	7,5302	7,5364	7,5427	7.5491	7,5556	7,5622	7,5690
99.5	7.5758	7,5828	7,5899	7,5972	7,6045	7,6121	7,6197	7,6276	7,6356	7,6437
99.6	7.6521	7,6606	7,6693	7,6783	7,6874	7,6968	7,7065	7,7164	7,7265	7,7370
99.7	7.7478	7,7589	7,7703	7,7821	7,7944	7,8070	7,8202	7,8338	7,8480	7,8627
99.8	7.8782	7,8943	7,9112	7,9291	7,9478	7,9677	8,9889	7,0114	8,1357	8,0618
99.9	8.0902	8,1214	8,1559	8,1947	8,2389	8,2905	8,3528	8,4316	8,5401	8,7190

ANNEX 3. Forms for recording data / results of fungicide tests.

7.1.1. 3.1 Form for recording benzimidazole fungicide sensitivity monitoring test

Sample no.	Sampling date:
Site:	Fungicide:
Farm:	Date and hour beginning incubation at 27ºC:
Field:	Date and hour beginning assessment:
	Date and hour end assessment:

	Concentration in ppm								
Spore No.	0			1			5		
	Normal	Not germinated	Abnormal	Normal	Not germinated	Abnormal	Normal	Not germinated	Abnormal
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
22									
23									
24									

	Concentration in ppm								
Spore No.	0			1			5		
	Normal	Not germinated	Abnormal	Normal	Not germinated	Abnormal	Normal	Not germinated	Abnormal
25									
26									
27									
28									
29									
30									
31									
32									
33									
34									
35									
36									
37									
38									
39									
40									
41									
42									
43									
44									
45									
46									
47									
48									
49									
50									
TOTAL									

7.1.2. 3.2 Form for recording ED_{50} and ED_{99} of Benzimidazole sensitivity test

Sample no.	Sampling date:
Site:	Fungicide:
Farm	Date and hour beginning incubation at 27ºC:
Field	Date and hour beginning assessment
	Date and hour end assessment

Ascospores classification	CONCENTRATION (PPM)						
	0	0.01	0.05	0.1	0.5	1	5
Normal germination							
Abnormal germination							
Not germinated							
TOTAL							

DE_{50}	
DE_{99}	
Regression equation	

7.1.3. 3.3 Form for recording DMI's sensitivity test

Sample no.	Sampling date:
Site:	Fungicide:
Farm	Date and hour beginning incubation at 27°C:
Field	Date and hour beginning assessment
	Date and hour end assessment

Length (µ) of *M. fijiensis* ascospore germ tubes (40x)

Spore no.	CONCENTRATION (PPM)						Spore no.	CONCENTRATION (PPM)					
	0	0.0001	0.001	0.01	0.1	1		0	0.0001	0.001	0.01	0.1	1
1							26						
2							27						
3							28						
4							29						
5							30						
6							31						
7							32						
8							33						
9							34						
10							35						
11							36						
12							37						
13							38						
14							39						
15							40						
16							41						
17							42						
18							43						
19							44						
20							45						
21							46						
22							47						
23							48						
24							49						
25							50						
TOTAL													

DE_{50}	
DE_{99}	
Regression equation	

7.1.4. 3.4 Form for recording distribution frequencies of growth inhibition of ascospore tube at different concentrations for DMI's sensitivity tests.

Sample no.	Sampling date:
Site:	Fungicide:
Farm:	Date and hour beginning incubation at 27°C:
Field:	Date and hour beggining assessment:
	Date and hour end of assessment:
Mean of germ tubes length at 0 µg/ml (control) after 48 hours incubation at 27°C:	

Classes of ascospore germ tube inhibition (%)	Range in µ with regard to germ tube growth in control	0.0001 ppm		0.001 ppm		0.01 ppm		0.1 ppm		1 ppm	
		Number spores in the range	%	Number spores in the range	%	Number spores in the range	%	Number spores in the range	%	Number spores in the range	%
1 (0%)	0										
2 (1-10 %)											
3 (11-30 %)											
4 (30-50%)											
5 (50-70%)											
6 (>70%)											
TOTAL											

7.1.5. 3.5 Form for recording summary of distribution frequencies of isolates in the range of DE_{50} of a site for DMI's sensitivity tests

Sampling date or sampling period:	
Samples no.	
Site:	
Farm(s)	
Field (s)	
Fungicide:	

Inhibition classes according DE_{50}		Number of isolates in the range	Frequency (%)
Classes	(DE_{50} in ppm)		
1	0.0001 - 0.0003		
2	0.0003 - 0.001		
3	0.001 - 0.003		
4	0.003 - 0.01		
5	0.01 - 0.03		
6	0.03 - 0.1		
7	0. 1 - 0.3		
8	0.3 - 1.0		
9	>1.0		
TOTAL			

7.1.6. 3.6 Form for recording AMINES sensitivity tests

Sample no.	Sampling date:
Site:	Date and hour beginning incubation at 27°C:
Farm	Date and hour beginning assessment:
Field	Date and hour end assessment:

Length (µ) of *M. fijiensis* ascospore germ tubes (40 x).

Spore no.	CONCENTRATION (PPM)						Spore no.	CONCENTRATION (PPM)					
	0	0.001	0.01	0.1	1	10		0	0.001	0.01	0.1	1	10
1							26						
2							27						
3							28						
4							29						
5							30						
6							31						
7							32						
8							33						
9							34						
10							35						
11							36						
12							37						
13							38						
14							39						
15							40						
16							41						
17							42						
18							43						
19							44						
20							45						
21							46						
22							47						
23							48						
24							49						
25							50						
TOTAL							TOTAL						

7.1.7. 3.7 Form for recording frequency distribution of growth inhibition of ascospore tube at different concentrations for AMINE sensitivity tests.

Sample no.	Sampling date:
Site:	Fungicide:
Farm	Date and hour beginning incubation at 27ºC:
Field	Date and hour beginning assessment
	Date and hour end of assessment

Classes of ascospore germ tube inhibition (%)	Range in µ in relation to germ tube growth in control	0.01 ppm		0.1 ppm		1 ppm		10 ppm	
		Number spores in the range	%	Number spores in the range	%	Number spores in the range	%	Number spores in the range	%
1 (0%)									
2 (1-10 %)									
3 (11-30 %)									
4 (30-50%)									
5 (50-70%)									
6 (>70%)									
TOTAL									

7.1.8. 3.8 Form for recording summary of distribution frequencies of isolates in the range of DE_{50} of a site for AMINES sensitivity tests.

Sampling date or sampling period:	
Samples no.	
Site:	
Farm(s)	
Field (s)	
Fungicide:	

Inhibition classes according DE_{50}		Number of isolates in the range	Frequency (%)
Classes	(DE_{50} in ppm)		
1	0.0001 - 0.0003		
2	0.0003 - 0.001		
3	0.001 - 0.003		
4	0.003 - 0.01		
5	0.01 - 0.03		
6	0.03 - 0.1		
7	0. 1 - 0.3		
8	0.3 - 1.0		
9	>1.0		
TOTAL			

7.1.9. 3.9. Form for recording QoI's sensitivity test

Sample no.	Sampling date:
Site:	Date and hour beginning incubation at 27ºC:
Farm	Date and hour beginning assessment:
Field	Date and hour end assessment:

Germination and length (µ) of *M. fijiensis* ascospore germ tubes (40x).

Spore	GERMINATION					Spore	Germ tube growth				
no.	0	0.05	0.1	1	5	no.	0	0.05	0.1	1	5
1						1					
2						2					
3						3					
4						4					
5						5					
6						6					
7						7					
8						8					
9						9					
10						10					
11						11					
12						12					
13						13					
14						14					
15						15					
16						16					
17						17					
18						18					
19						19					
20						20					
21						21					
22						22					
23						23					
24						24					
25						25					
TOTAL						TOTAL					

8. BIBLIOGRAPHY

Anon. 1980. A renewed challenge: Black and Yellow Sigatoka. Monitoring for possible tolerance to benzimidazoles. Field and Laboratory procedures. *Dupont.*

Avila-Adame, C. and Köller W. 2003. Characterization of spontaneous mutants of *Magnaporthe grisea* expressing stable resistance to the Qo-inhibiting fungicide azoxystrobin. *Curr. Genet.* 42: 332–338

Balashov, S.V., Gardiner, R., Park, S. and Perlin, D.S. 2005. Rapid, high-throughput, multiplex, real-time PCR for identification of mutations in the *cyp 51A* gene of *Aspergillus fumigatus* that confer resistance to itraconazole. *J. Clin. Microbiol.* 43: 214–222.

Bliss, C. I 1935 The calculation of the dosage mortality curve. *Annals of Applied Biology* 22:134-167.

Bollen, G.J., 1981. Fungicide resistance and microbial balance. In Fungicide Resistance in Crop Protection. (Ed.) J. Dekker y S Georgopoulos. Editorial PUDOC Wageningen. 265pp.

Brasseur, G., Saribas, A.S. and Daldal, F., 1996. A compilation of mutations located in the cytochrome b subunit of the bacterial and mitochondrial bc1 complex. *Biochim. Biophys. Acta,* 1275:61–69

Buchenauer, H. 1995. DMI fungicides-side effects on the plant and problem of resistance. In: Modern Selective Fungicides Ed. Prof Horst, Lyr. Gustav Fischer, Verlag. New York, Pp 259-290.

Cañas-Gutiérrez, G.P., Angarita-Velásquez, M.J. Restrepo-Flórez, J.M., Rodríguez, P., Moreno, C.X. and Arango, R., 2009. Analysis of the CYP51 gene and encoded protein in propiconazole - resistant isolates of *Mycosphaerella fijiensis. Pest Manag. Sci.* 65: 892–899

Chaleff, R.S. and Mauvais, C.J., 1984. Acetolactat synthase is the site of action of two sulfonyl urea herbicides in higher plants. *Science* 244,: 1443-1445.

Chin, K. M., Wirz, M., and Laird, D. 2001. Sensitivity of *Mycosphaerella fijiensis* from banana to trifloxystrobin. *Plant Dis.* 85:1264-1270

Davidse, L.C., 1981. Benzimidazole compounds: selectivity and resistance. In: Fungicide Resistance in Crop Protection. (Ed.) Dekker J. and Georgopoulos, S. Editorial PUDOC Wageningen, 265 pp.

Davidse, L.C. and Ishii, H. 1995. Biochemical and molecular aspects of the mechanisms of action of benzimidazoles, N phenylcarbamates, N phenylformamidoximes and the mechanisms of resistance to these compounds in fungi. In: Modern Selective Fungicides Ed. Prof . Horst, Lyr. Gustav Fischer, Verlag. New York. Pp 305 – 322.

Duvert, D.; Milling, R. and González Quesada, R. 2002. Monitoring the sensitivity of *Mycosphaerella fijiensis* to pyrimethanil. Proceedings XV meeting] - AUGURA, Asociación de Bananeros de Colombia, Medellín (COL) - p. 153-157.

Edginton, L.V., 1981. Structural requirements of systemic fungicides. *Ann. Rev, of Phytopathology* 19, 107-124.

Esau, K., 1965. Plant anatomy, 2nd, Edition, John Wiley and Sons, New York- London-Sidney.

FAOStat. 2013. http://faostat3.fao.org/home/index.htm l

Fouré, E. 1982. La maladie des raies noires des bananiers et plantains *M. fijiensis* (Morelet) Etude comparée des différents symptômes et stades de la maladie au Gabon IRFA-CIRAD-France et SONADECI-Gabon

Fouré, E., Grisoni, M. et Zurfluh, R. 1984. Les Cercosporiose du bananier et leur traitement. Comportement des variétés. Etude de la sensibilité variétale des bananiers et plantains a *Mycosphaerella fijiensis* Morelet et des quelques caractéristiques biologiques de la maladie des raies noires au Gabon. *Fruits* 39 (6): 365 - 378.

FRAC, 1991. Methods for monitoring fungicide resistance developed by the working groups of the Fungicide Resistance Action Committee (FRAC) of GIFAP. *Bulletin OEPP* 21 (2): 291-354,

FRAC, 2007. Mode of Action of Fungicides. FRAC classification on mode of action 2007. (www.frac.info)

Fraaije, B.A., Cools, H.J. , Kim, S.H., Motteram, J., Clark ,W.S. and Lucas, J. A. 2007. A novel substitution I381V in the sterol 14a-demethylase (CYP51) of *Mycosphaerella graminicola* is differentially selected by azole fungicides

Geier B., Schnagger, H., Brandt, U., Colson, A. M., and Von, Jagow, G., 1992. Point mutation in cytochrome b of yeast ubihydroquinone: cytochrome–c oxidoreductase causing myxothiazol resistance and facilitated dissociation of the iron–sulfur subunit. European *Journal of Biochemistry* 208 (2): 375 – 380.

Georgopoulos, S.G., 1981. Cross-resistance. Fungicide resistance in Crop Protection (ed) by I. Dekker and S.G. Georgopoulos. Wageningen. pp. 53-59,

Gisi U., Sierotzki H., Cook, A. and McCaffery, A. 2002.Mechanisms influencing the evolution to Qo inhibitor fungicides. *Pest Management Science* 58: 859-867.

Godwin, J.R., Anthony, J.M., Clough, J.M., Godfrey, C.R.A., 1992. ICI 5504: a novel, broad spectrum systemic (beta-methoxyacrilate fungicide). Proceedings of the Brighton Crop Protection Conference on Pest and Diseases, Vol. 1, pp. 435–442.

Hassall, K.A., 1992. The chemistry of pesticides, their metabolism, modes of action and uses in crop protection. Verlag Chemie, Weinheim – Deerfield – Beach – Florida - Basel.

Heaney, S.P., Hall, A.A., David, S.A. and Olaya, G. 2000. Resistance to fungicides in the Qol- STAR cross resistance group: current perspective. Proceedings of the BCPC Conference Pest and Diseases 2000, 755 – 762.

Jackson, L.L. and Frear, D.S., 1968. Lipids of rust fungi. II. Stigmast – 7-enol and stigmasta-7,24(28)-dienol in flax rust uredospores. *Phytochemistry* 7: 65.

Joseph-Horne, T., Hollomon, D.W., Wood, P.M. 2001. Fungal respiration: a fusion of standard and alternative components. *Biochim. Biophys. Acta* 1504:179–195

Kim, Y.S., Dixon, E.W., Vincelli, P. and Farman, M.L 2003. Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome b gene. *Phytopathology* 93:891-900.

Koeller, W. 1987. Plant growth regulator activities of stereochemical isomers of triadimenol. *Physiol. Plant* 71: 309.

Köller, W. and Scheinpflug, H., 1987. Fungal resistance to sterol byosinthesis inhibitors: a new challenge. *Plant Disease* 71: 1066 - 1074.

Köller, W., 1999. Chemical approaches to managing plant pathogens. In: Ruberson, J.R. (ed) Handbook of pest management. Dekker, New York, pp 337–376.

Kuck, K.H. and Mehl, A., 2003. Trifloxystrobin: resistance risk and resistance management. *Planzenchutz-Nachrichten Bayer* 56 (74): 313-325.

Kuck, K.H., Scheinpflug, H., and Pontzen, R. 1995. DMI fungicides. In: Modern Selective Fungicides Ed. Prof Horst, Lyr. Gustav Fischer, Verlag. New York, Pp 205 – 258.

Lescot, T. 2011. La diversité génétique des bananiers, *FruiTrop* 89 : 58-62.

Loeffler, R.S.T., Butters, J.A. and Hollomon, D.W. 1984. The lipid contents of powdery mildews and their sensitivity to fungicides. *Proc.1984 Brit. Crop Prot. Conf*. : 911.

Loeillet, D. (2005). Le commerce international de la banane : entre évolution et révolution, *Frui-Trop,* Vol.129: 2–19.

Lyr, H. 1995. Selectivity in modern fungicides and its basis. In: Modern Selective Fungicides Ed. Ed., Lyr, H. Gustav Fischer, Verlag. New York. P. 13–22.

Mather K., 1953. The genetical structure of populations. *Symposia of the Society for Experimental Biology 7*. Cambridge University Press, p. 66-95

Margot, P., Huggenberger, F., Amrei, J. and Weiss, B., 1998. CGA 279202, a new broad-spectrum strobilurin Fungicide. Brighton Crop Protection Conference: Pest & Diseases 1998: Volume 2: Proceedings of an International Conference, Brighton, UK, 16 – 19 November 1998: 375 – 382.

Mourichon, X. et Zapater, M.F. 1990. Obtention in vitro du stade *Mycosphaerella fijiensis*, forme parfaite de *Cercospora fijiensis*. Fruits 45 (6): 553-557.

Muiño, B., Pérez, L. and Iglesias, M., 1993. Reduced sensitivity to propiconazole in *M. musicola*. Proceedings VII International Congress of Plant Pathology, Montreal. July 28th -August 5.

Münch, E. 1930. Die Stoffbewegungen in der Pflanze. Gustav Fischer, Jena.

Neumann, St. and Jacob, F., 1995. Principles of the uptake and systemic transport of fungicides within the plant. In: Modern Selective Fungicides Ed. Prof Ed., Lyr, H. Gustav Fischer, Verlag. New York. Pp. 54-73

Pérez, L., 1980. Morfología, distribución, aspectos bioecológicos y lucha contra *Mycosphaerella musicola* Leach, agente causal de la Sigatoka de los plátanos. Tesis para aspirar al título de Doctor en Ciencias Agrícolas. Instituto Superior de Ciencias Agropecuarias de la Habana.

Pérez, L., 1993. Caracterización morfológica y cultural del patógeno causante de la Sigatoka negra (*Mycosphaerella fijiensis* Morelet). (Informe no publicado) Instituto de Investigaciones de Sanidad Vegetal. INFORME FINAL 003.14.12. 26pp.

Pérez, L. 1998. Sigatoka negra (*Mycosphaerella fijiensis* Morelet) de bananos y plátanos (*Musa spp*.) en Cuba. Biología, epidemiología y manejo integrado de la enfermedad. En: Robles, M.; Vizcaino I., Orozco, J., González, L.A., Zapata, R.J., Garza, G., Memorias Simposio Internacional Sigatoka negra. Manzanillo, Colima, México. 8-10 de Julio. Pp. 24–52.

Pérez, L. 2002. Morfología de las especies de *Mycosphaerella* asociadas a manchas de las hojas en *Musa spp. Fitosanidad* 6(2): 3-9.

Pérez, L., Iglesias, M. y Mauri, F., 1985. Aparición de una raza resistente al benomyl de *Mycosphaerella musicola*, agente causal de la Sigatoka en el plátano. *Agrotecnia de Cuba* 17 (1): 79-88,

Pérez, L. y Battle, A. 1993. Procedimiento de monitoreo para determinar la sensibilidad de *Mycosphaerella spp.* en bananos y plátanos a fungicidas inhibidores de la síntesis de ergosterol (IBE) y particularmente al propiconazol, otros triazoles y al tridemorph. Instituto de Investigaciones de Sanidad Vegetal. MINAG.

Pérez, L, Batlle, A., Hernández, A., Trujillo, R., Alvarez, C and Méndez, A. 2003. Evolución de la sensibilidad a fungicidas de las poblaciones de *Mycosphaerella fijiensis* Morelet en banano en Cuba. *Fitosanidad* 7(3): 49-54

Pérez, L., Hernández, A., Hernández, L., and Pérez, M., 2002. Effect of trifloxystrobin and azoxystrobin on the control of black Sigatoka *(Mycosphaerella fijiensis* Morelet) on banana and plantain. *Crop Protection* 21: 17 -23.

Pérez, L., Iglesias, M. y Mauri, F., 1985. Aparición de una raza resistente al benomyl de *Mycosphaerella musicola*, agente causal de la Sigatoka en el plátano. *Agrotecnia de Cuba* 17 (1): 79-88,

Pérez, L. y Mauri, F., 1981. Un método simple para detectar resistencia al carbendazim. *Ciencia y Técnica en la Agricultura. Serie Protección de Plantas* 5 (3): 65 – 91

Pérez, L. and Mauri, F., 1994. Efficacy of EBI fungicides on the control of Sigatoka disease of bananas caused by *Mycosphaerella musicola* Leach ex Mulder in Cuba. *International Journal of Pest Management* 40 (1): 1- 5.

Pérez, L., Mauri, F., Barranco, B. and García, G., 1993. Efficacy of EBI's fungicides in the control of *Mycosphaerella fijiensis* Morelet of bananas and plantains with treatments based on stage of evolution of the disease (biological warnings) in Cuba. Proceedings VII International Congress of Plant Pathology. Montreal. Jul 28 - Ago 5.

Pérez, M., Rebullido, R. y Pérez, L. 2004. Líneas base de sensibilidad de las poblaciones de *Mycosphaerella fijiensis* Morelet de Cuba, a los fungicidas azoxystrobin y trifloxystrobin. *Fitosanidad* 8 (3): 41-47.

Pommer, E.H., Otto, S. and Kradel, J. 1969. Some results concerning the systemic action of tridemorph, *Proc. 5th Brit. Insec. Fung. Conf.:* 347-353.

Pont, W., 1960. Three leaf speckle diseases of the banana in Queensland. *Queensl. J. Agr. Sci.* 17, 273- 309.

Russell, P.E., 2007. Sensitivity baselines in fungicide resistance Research and management. FRAC Monograph No. 3. Crop Life International. Pp. 58. Great Britain

Sierotzki, H., Wullschleger, J. and Gisi, U., 2000a. Point mutation in cytochrome b gene conferring resistance to strobilurine fungicides in *Erysiphe graminis f. sp. tritici* field isolates. Pest Biochem. Physiology 68, 107-112.

Sierotzki, H., Parisi, S., Steinfeld, U., Tenser, I., Poirey, S. and Gisi, U., 2000b. Mode of resistance to respiration inhibitors at the cytochrome bc_1 complex of *Mycosphaerella fijiensis*. Pest Management Science 56, 833-841.

Sisler, H.D., 1996. I. Chemistry and form of action of inhibitors of biosynthesis of sterols. II. Characteristics of the fungal resistance to 14 α- demetilase inhibitors (DMIs). Presented at 4th Meeting of Banana Group of the Fungicide Resistance Action Committee (FRAC). Miami.

Smith, C.M., Trivellas, A.E., Johnson, L.E.B., and Joshi, M.M., 1991. Methods for monitoring the sensitivity of a range of fungal pathogens to benzimidazol fungicides. In: FRAC Methods for monitoring fungicide resistance. *EPPO Bull.*21, 336-341.

Stahel, G., 1937. Notes on the Cercospora leaf spot of bananas (*Cercospora musae*). Trop. *Agric. Trin*. 14, 257 - 264.

Stover, R.H., 1963. Leaf spot of bananas caused by *Mycosphaerella musicola*: associated ascomycetous fungi. *Canadian Journal of Botany*, 41: 1481-1485.

Stover, R.H. (1969). The *Mycosphaerella* species associated with banana leaf spots. *Trop. Agric. Trin*. 46, 325 - 331.

Stover, R.H. (1972). Banana, plantain and abaca diseases, Commonw. Mycol. Inst., Kew, Surrey, UK

Stover, R.H., 1977. A non-virulent benomyl tolerant *Cercospora* from leaf spots caused by *Mycosphaerella fijiensis var. difformis* and *M. musicola*. *Trans. Brit. Mycol. Soc*. 69, 500 - 502.

Stover, R.H., 1994. *Mycosphaerella musae* and *Cercospora* "non-virulentum" from Sigatoka leaf spots are identical. *Fruits* 49, 187-190.

Vincent, P.G., and Sisler, H.D. 1968. *Physiol. Plantarum* 21, 1249.

Wolfe, M.S., 1981. Dynamics of pathogen population in relation to fungicide resistance. In: Fungicide resistance in crop protection. (Ed) by J. Dekker and S.G. Georgopoulos. Wageningen. Pp 46-52

Wyand, R. and Brown, J., 2005. Sequence variations in the CYP51 gene of *Blumeria graminis* associated with resistance to sterol demethylase inhibiting fungicides. *Fungal Genet. Biol.* 42:726–735.

Zheng, D., Olaya, G., and Koller, W. *2000.* Characterization of laboratory mutants of *Venturia inaequalis* resistant to the strobilurin-related fungicide kresoxim-methyl. Curr. Genet. 38:148-155.

Ziegler, H., Benet-Buchholz, J., Etzel, W. and Gayer, H., 2003. Tryflozystrobin a new strobilurin fungicide with and outstanding biological activity. Planzenchutz-Nachrichten Bayer 56 (74): 213-230.