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REVIEW ARTICLE

# New insights into fungicide resistance: a growing challenge in crop protection

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## Abstract

Development of fungicide resistance in plant pathogens is a challenging problem that has affected the performance of several site-specific fungicide groups including benzimidazoles, phenylamides, demethylation inhibitors, quinone outside inhibitors and succinate dehydrogenase inhibitors. Over the past years, research efforts have led to a better understanding of the emergence, spread, behaviour, diagnostics and mechanisms of resistance to different groups of fungicides in diverse types of pathogens. Molecular tools have proved handy in rapid detection of resistance that has greatly helped in monitoring the evolution of resistance in pathogen populations. The recently introduced unified system of labeling resistance-associated mutations has made it easier to determine novel changes in amino acids of the target protein. Knowledge of fitness cost of resistance and risk assessment is crucial in developing resistance management guidelines. Discovery of novel modes of action is an important aspect of resistance management. Use of different modes of action including conventional multi-site inhibitors in mixtures with at-risk fungicides still appears relevant to avoid resistance build up. There is a growing focus on the use of biologicals including biocontrol agents as a part of resistance management programmes.

**Keywords** At-risk fungicides · Evolution of resistance · Fitness costs · Novel modes of action · Resistance management · Risk assessment · Target-site mutations

## Introduction

Fungicide compounds, despite some limitations, have become an integral part of the present-day crop protection aimed at getting desirable yields. The fact is that several devastating fungal diseases can be effectively controlled only by the sole use of fungicides, as genetic host resistance is rarely available or is unstable and other means including antagonistic microbes fall short of providing adequate protection. The modern fungicides are ecologically safer and are used at much lower dose rates as compared to the earlier compounds. Nevertheless, most of the fungicides developed after 1970 are selective in their biochemical modes of action and are prone to the development of resistance in target pathogens.

Fungicide resistance has become a serious problem with the use of site-specific fungicides the world over. It has affected the performance and active life of some highly promising fungicides, and in many instances, it has led to failure of disease control (Brent 1995). Ever since the introduction of benzimidazoles, most of the selective fungicides have been known to carry varying levels of resistance risk. There has been an increasing awareness about the problem of resistance over the past four decades and it has become a major focus in fungicide research (Thind 2021). In fact, fungicide resistance is now considered as a major challenge in the discovery and development of novel modes of action (Hollomon 2015). It has not only affected the farmers with low economic returns due to yield loss but also the manufacturers and the consumers alike.

Researchers over the years have focused efforts in understanding various aspects of fungicide resistance in pathogens of concern. As a result, headways have been made in our knowledge on the occurrence, spread, behaviour, diagnostics and mechanisms of resistance to different groups of fungicides in diverse types of pathogens with better strategies to manage the problem. This review analyses the threat of fungicide resistance in crop protection as we know it today

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and briefs about new insights into the problem unraveled in recent years.

## Resistance not the sole cause of reduced fungicide efficacy

There are some misconceptions about the role of fungicide resistance in disease control failures that still exist today. It is generally considered that poor disease control with fungicide applications is due to development of resistance in the pathogens. Many a times, fungicide failures are not due to resistance build up. One needs to rule out other possible causes of inferior disease control on using a fungicide before attributing it to the development of resistance. The other reasons of reduced control are linked to fungicide application (e.g., inadequate dose, improper timing, poor spray coverage, wrong choice of fungicide, wrong tank mixture partner), environmental conditions (e.g., conditions highly favourable for rapid disease development and spread, fungicide residue wash off by rain, losses from leaf surface through weathering, excessively wet or dry soil) and plant characteristics (e.g., new plant parts not protected due to fast growth, dense plant canopy). Resistance develops through natural selection of mutants in a pathogen population under fungicide pressure and is an inheritable change in the ability of fungal pathogens to survive a fungicide. Nevertheless, any occurrence of resistance needs to be confirmed following standardized procedures before reporting new cases.

## Resistance scenario—past to present

Conventional multi-site fungicides such as dithiocarbamates, copper compounds, phthalimides, etc. did not face the problem of resistance even after their prolonged use in several crops and this holds true even today. However, with the introduction of site-specific systemic fungicides beginning with benzimidazoles in the late 1960s, that were able to provide much superior control of different plant diseases than contact fungicides, first development of resistance to benomyl was reported in cucurbit powdery mildew after only two years of commercial use in the USA (Schroeder and Provvidenti 1969). This was quickly followed by other reports of resistance to benomyl and related compounds in the pathogens causing apple scab, peanut leaf spot, grey mold of grape and other crops, green and blue molds of citrus, cereal eyespot, cucurbit and barley powdery mildews, etc. in 1970s from Europe and USA. Simultaneously near the same time, reports of resistance to dodine (*Venturia inaequalis*), dimethirimol (cucurbit powdery mildew), ethirimol (barley powdery mildew), kasugamycin and iprobenphos (*Pyricularia oryzae*) were also published (Dekker and Georgopoulos 1982; Russell 1995). Subsequently, dicarboximides met with similar fate soon after their introduction and resistance developing quite rapidly in *Botrytis* and related fungal species. Triazoles along with other DMI fungicides are the largest group of fungicides introduced in 1970s and early 1980s. These compounds provided unaffected control of several important diseases in field crops, fruits, vegetables and plantation crops for several years of their use. Low to moderate resistance build up was reported in many fungal pathogens after 8–10 years of their use. These are still being widely used alone or in combination with other modes of action (Koller and Scheinpflug 1987).

When phenylamides (notably acylalanines) were introduced in late 1970s, these were considered to be landmark discovery for the control of oomycete pathogens. Their most promising representative, metalaxyl, provided much needed effective control of downy mildews and Phytophthora blights in a variety of crops in the beginning. However, after merely 1–2 years of its introduction, resistance cases were reported in several countries (Davidse et al. 1981). The problem continued to affect several crops and these were later introduced in combination with mancozeb and other multisite fungicides (Gisi 2002).

Similar situation was observed with strobilurins which were introduced as ecofriendly compounds with wide spectrum of disease control, starting with azoxystrobin and kresoxim methyl in mid-1990s. Resistance developed quickly to strobilurins and other QoI fungicides in several pathogens after 2–3 years of their use on a range of crops. This prompted the manufacturers to develop these fungicides as combination products (Leadbeater 2012). Resistance to quinoline fungicides, released in late 1990s for control of powdery mildews, developed at slow pace and reports of low level of resistance to quinoxyfen in cereal and grape powdery mildews appeared much later. SDHI fungicides (boscalid, fluopyram, fluxapyroxad), which are used in a variety of crops against a wide spectrum of diseases, are too suffering from loss of sensitivity in pathogens like *Botrytis cinerea, Sclerotinia homoeocarpa* and more recently in *Podosphaera xanthii* and *Blumeriella jaapii* (Fernandez-Ortuno et al. 2017; Gleason et al. 2021). Similarly, moderate resistance has been reported in *B. cinerea* to fludioxonil (phenylpyrroles) and cyprodinil (anilinopyrimidines) during last decade (Hahn 2014). Contrarily, in case of CAA group fungicides (iprovalicarb, mandipropamid, dimethomorph), generally applied to control oomycete pathogens, no serious issues of resistance emerged and these are delivering normal efficacy under practical situations, except that low level of resistance to mandipropamid has recently been observed in some grape downy mildew populations in Italy (Toffolatti et al. 2018). Similar is the case of benzamides with fluopicolide resistant isolates detected recently in grape and cucurbit downy mildews in USA.

No practical loss of sensitivity has been recorded so far in case of recently introduced anti-oomycete fungicides, oxathiapiprolin and fluoxapiprolin belonging to the group piperidinyl thiazole isoxazolines. It is noteworthy to add that resistance to benzimidazoles, DMI and strobilurin fungicides has also been reported in some fungal parasites of cultivated mushrooms (Gea et al. 2021). Overall, cases of resistance to many of the above mentioned site-specific fungicides have increased steadily over the past five decades in different countries and many among these have led to partial or complete disease control failures in key crops. Till now, resistance in more than 230 fungal plant pathogens has been reported against more than 100 active ingredients in different crops and geographical regions (FRAC 2020).

## Dynamics of emergence and evolution of resistance

Resistance emerges and evolves through natural selection of resistant mutants in the field populations of a pathogen with a history of extensive use of the fungicide in question that fails to give expected level of disease control with its application at recommended rate. Resistant strains arise through genetic mutations or natural variations in fungal populations which then build up as sub-populations in the otherwise sensitive populations of the pathogens. The resistant strains then evolve further with selection pressure of site-specific fungicides and their repeated applications lead to a marked shift in the sensitivity levels of pathogen populations. The fungicide efficacy is impaired when resistant fungal propagules predominate over sensitive propagules in a population.

Emergence and evolution of resistance are influenced by the nature of pathogen and fungicide properties (Hollomon 2015). Resistance emerges rapidly in pathogens with short reproduction time, profuse sporulation with relative abundance of genotypes with varying sensitivities, rapid dispersal through wind, and ability to infect most plant parts. Likewise, nature of the fungicide is an important factor in emergence of resistance. Repeated and exclusive use of a site-specific fungicide, better solubility and rapid absorption and distribution in the plant system, large extent of treated area with the same fungicide are some of the properties that can favour emergence and selection of resistant strains. While in case of some site-specific fungicides such as benzimidazoles, phenylamides or strobilurins, a high level of resistance (qualitative) can evolve as a result of single point mutation in the target protein resulting in two distinct populations (bimodal distribution), in others like DMIs where more than one allele confers resistance (quantitative), unimodal distribution is observed with gradual multi-step shifts towards resistance over several crop seasons.

Various theoretical models on evolution of resistance have been worked out in the past and lately the emphasis has shifted to realistic modelling linked to field situations. Taking *Mycosphaerella graminicola* on winter wheat as a case study, Hobbelen et al. (2014) have derived a model, based on emergence time of resistant strains for a range of mutations and fitness costs of resistance, to describe emergence and evolution of resistance in a sensitive pathogen population. In another study in Europe, with focus on four cereal pathogens (eye spot, Septoria blotch, powdery mildew, and Fusarium ear blight) and major fungicide classes used for their control (MBCs, DMIs, QoIs, SDHIs), knowledge of underlying mechanisms of resistance and their genetic control has been used to explain emergence of resistance and its impact on disease management (Lucas et al. 2015). More recently, Massi et al. (2021) have discussed different phases of fungicide resistance evolution in *Plasmopara viticola* under the selection pressure of different groups of fungicides viz. phenylamides, CAA, QoI, QiI, and benzamides based on extensive monitoring through several seasons. They emphasized the need to employ multiple testing procedures to get realistic view of resistance evolution. In a case study with *Phytophthora infestans*, potato fields having cultivars of six diverse genetic background were found to influence the evolution of resistance to azoxystrobin and increased its sensitivity to *P. infestans* (Yang et al. 2021). Such studies are useful to evaluate the impact of anti-resistance strategies in the field.

## Monitoring and detection: from bioassays to molecular tools

Regular monitoring for detecting sensitivity shifts in a pathogen population under fungicide treatments serves as an early warning system for signs of impending resistance build up. Monitoring is a crucial aspect of resistance research because basically all our knowledge on the evolution, distribution and impact of resistance in the field has been gained through extensive monitoring. Monitoring is also essential to assess the effectiveness of anti-resistance strategies employed. Different sampling methods are followed depending on the disease and the host crop and these range from driving a vehicle with test plants on its top through the cropped areas (for measuring the sensitivity response of whole pathogen populations) to taking the representative single pustules and even single spore isolates to estimate distribution of resistant individuals (Brent 1992). Generally, freshly sporulating lesions are collected for use in sensitivity assays.

Ever since 1970s and 1980s, when early cases of resistance to benzimidazoles, pyrimidines and DMIs started appearing, a range of standard laboratory method had been in use for detection of fungicide resistance in target

pathogens. The commonly used bioassays include spore germination rate or spore germ-tube length tests and mycelial growth tests on fungicide amended nutrient media (for culturable pathogens) and treated leaf disc or detached leaf tests (for obligate pathogens). These assays involve exposure of the fungal spores/mycelia to a single discriminatory concentration or more commonly to a range of fungicide concentrations and then calculation of resistance factor (RF) by comparing the inhibitory values ($ED_{50}$) of test strains with that of sensitive strain. Careful determination of baseline sensitivity of the unexposed, sensitive pathogen population to at-risk fungicide and calibration of discriminatory dose is the most crucial step in detection of resistance. However, these bioassays are time-consuming and take three to more than seven days before we get the results. As a substitute to linear mycelial growth method, an automated quantitative assay using microplate reader for measuring fungal growth was developed by Roposo et al. (1995) to determine iprodione resistance in *Botrytis cinerea*. This microtiter assay uses absorbance in the range of 0.0–0.6 units as a measure of fungal growth, requires less time and 96 samples can be processed at a time. The same method was later used by others to test sensitivity of *B. cinerea* to boscalid. Mycelial growth assays using fungicide-amended synthetic medium are not always reliable for resistance monitoring as claimed by Ishii et al. (2021) while working on DMI resistance in *Venturia nashicola* causing Asian pear scab in Japan.

With gain in knowledge of the biochemical mechanisms of resistance and identification of changes in DNA, monitoring research saw a major push with the availability of PCR-based molecular tests in late 1990s. Detection and quantification of point mutations in pathogen populations have now become a routine practice in fungicide resistance research. Starting with strobilurins, point mutations in DNA have been identified in all major pathogens having developed resistance to different groups of at-risk fungicides (Fraaije et al. 2002). Various nucleic acid–based techniques developed for detection of resistance to different groups of site-specific fungicides viz. benzimidazoles, dicarboximides, sterol biosynthesis inhibitors, QoI, SDHI, anilinopyrimidines and phenylpyrroles have been discussed by Beckerman (2013).

In a study with *Blumeria graminis* f. sp. *hordei,* Zulak et al. (2018) used more efficient digital PCR assay for detection and quantification of two mutations (Y136F and S509T) in the Cyp51 gene that confers resistance to DMI fungicides. Allele specific RT-PCR and droplet digital PCR have been used for molecular detection of resistance to QoIs, Qils and CAAs in *Plasmopara viticola*. In the grape downy mildew pathogen, QoI resistance is associated with mutations F129L or G143A, while resistance to CAAs is associated with several SNPs in the third gene of cellulose synthase. For simultaneous detection of QoI and CAA resistant mutants in *P. viticola* population, the amplification-refractory mutation system PCR assay (ARMS) has been developed with improved accuracy (Massi et al. 2021). In China, Huang et al. (2020) has developed TaqMan-MGB real-time PCR assay to assess the frequency of CAA resistant alleles in *P. viticola* population in a vineyard. More recently, a rapid in-situ ASqPCR assay has been developed for quantification of strobilurin resistance mutation G143A in wheat powdery mildew fungus *B. graminis* f. sp. *tritici* (Dodhia et al. 2021). This in-field assay can detect resistant alleles in 90 min after sample collection.

Various target-site mutations leading to substitutions in the amino acid sequence of the target protein and thereby conferring resistance are mentioned in Table 1 for the major classes of at-risk fungicides.

These nucleic acid-based diagnostic techniques are rapid and more sensitive and have also proved useful in following the evolution of resistance in a pathogen population. By using whole genome sequencing data and association mapping of a global collection of wheat blotch pathogen *Parastagonospora nodorum*, Pereira et al. (2020) were able to reveal multilocus genetic architecture of resistance to azole fungicides and recapitulate the emergence of resistance. They found distinct combinations of resistance genes that evolved in the populations and identified 34 SNPs in close proximity to genes associated with resistance in other fungi.

**Table 1** Common target-site mutations conferring resistance to major fungicide classes

| Fungicide class | Target protein | Mutations |
|---|---|---|
| Quinone outside inhibitors | Ubiquinol oxidase | G143A, F129L in *cyt b* gene |
| Succinate dehydrogenase inhibitors | Succinate dehydrogenase | H/Y (or H/L) at 257, 267, 272 or P225F, H267L in *sdh* gene |
| Dicarboximides | MAP/Histidine-kinase | Mostly I365S, V368F, F267L in os-*1* gene |
| Benzimidazoles | ß-tubulin assembly | E198A/G/K, F200Y, L240F in β-tubulin gene |
| Demethylation inhibitors (DMIs) | C14-demethylase | V136F, Y137F, A379G, I381V in *cyp51A, cyp51B* gene |
| Carboxylic acid amides | Cellulose synthase | G1105S, V1109L in gene *cesA3* |
| Phenylamides | RNA polymerase | V1476G, P980S in *rpa190* gene |

Main source: Mair et al. (2016)

An interesting field kit-supported programme for resistance monitoring in peach brown rot fungus *Monilinia fructicola* using 24-well plates containing fungicide amended medium inoculated by fungal spores from peach fruits has been developed by Schnabel et al. (2012) in South Carolina. A real-time online web application created a sensitivity profile for an orchard on entering the pathogen growth data from well plates. This web application addresses needs of individual growers and helped with customized fungicide programmes for the growers. The same research group later came up with a smartphone app MyIPM in 2019 for fruit growers for early warning to avoid resistance build up.

Starting in 1991, FRAC (International) has published resistance monitoring and detection methods for various at-risk fungicide classes and for a range of important pathogens. These are available on FRAC web-site www.frac.info and have proved handy for resistance workers.

## Unified nomenclature of target-site mutations

It is well documented that development of resistance to fungicides is often associated with substitutions in the amino acid sequence of the target protein. For describing amino acid substitutions, the convention so far has been to cite the wild type amino acid, the codon number and the new amino acid using the one-letter amino acid code (e.g., alanine (A) for glycine (G) substitution in the cytochrome b gene at position 143 conferring resistance to strobilurin fungicides, referred to as G143A). Amino acid substitutions at the target site have been described for seven classes of at-risk fungicides. These, as per FRAC code, are C3, cytochrome b (cytb); G1, C-14 demethylases (Cyp51A, Cyp51B); B1/B2, *b*-tubulin: C2, succinate dehydrogenase complex (SdhB, SdhC, SdhD): H5, cellulose synthaseA3 (CesC3); E3, Os1 family (group III) histidine kinase (Os-1); and G3, 3-keto reductase (Erg27). In the present nomenclature of mutations, it has been observed that though the orthologous amino acid mutations have been selected in different fungal species from the same mode of action class, but the amino acids have different numbers. For example, Cyp51B amino acid Y137 in *Zymnoseptoria tritici* is orthologous to amino acid numbers 131–145 in different species. Likewise, in *Pyrenophora teres*, SdhB amino acid H277 is orthologous to amino acid numbers 249–278. This difference in numbering is because of different length of target proteins in each species, that creates undue confusion and masks relationship between mutations in different species.

To streamline the system of naming target-site mutations, Mair et al. (2016) have proposed a system for unifying the labelling of amino acids in target proteins. For this, they have produced a set of alignments between target proteins of relevant species fitted to a well-studied 'archetype' species. It is proposed that the orthologous amino acids in all species, presumed to be descended from the same amino acid, are given the same number irrespective of the actual position. In other words, same 'mutation label' will be assigned to orthologous mutations. In order to avoid confusion, mutation labels are to be italicized and mutation numbers should use regular lettering. With unified system of mutation labelling, it will be much easier to identify important changes in codon, and to determine whether these are new changes or just the same already observed in other species.

## Resistance risk assessment

Early assessment of resistance risk of a new fungicide is an important component for developing resistance management guidelines. For each fungicide with novel mode of action, it is now mandatory to submit information on resistance risk assessment while submitting dossier for registration to the regulatory authorities.

It is well known that profusely sporulating pathogens with short generation time and going through sexual recombination have more chances of resistance development, as these traits provide high genetic diversity and more chances of mutation in a population, leading to reduced sensitivity to the fungicide in use. Similarly, fungicides having single-site mode of action (viz. phenylamides, QoI fungicides) appear to have a higher risk of resistance, while fungicides with multiple modes of action (viz. dithiocarbamates, copper compounds) have quite low risk for resistance to develop.

There are several ways to assess resistance risk of new fungicides and requires integration of many factors. The initial step is to establish if there is cross resistance in target pathogens known to have resistance to existing fungicides in use. A new fungicide with structural difference but same mode of action as the existing analogues in the group is considered to carry similar resistance risk (e.g., DMIs, QoIs). As fungicide resistance is a phenomenon of natural selection, target pathogen's potential to generate resistant mutants is a major factor in risk assessment. Inherent risk of new mode of action is analyzed by generation of resistant mutants either by using mutagens or by exposing the pathogen to increasing fungicide concentrations on culture media. Risk analysis is greatly influenced by stability of resistant mutants. These days, a range of molecular and recombinant DNA techniques together with protein modelling and crystallography are used in resistance risk analysis that can predict the impact of amino acid changes on resistance (Frey et al. 2010).

However, laboratory generation of resistant mutants cannot always be correlated with development of resistance in field. In actual crop situation, resistance to fungicides

emerges from individual mutations in a pathogen population and its extent is driven by a combination of traits linked to pathogens, fungicides and agronomic systems. Using a data set of 67 European cases of resistance to single-site fungicides, Grimmer et al. (2014) prepared a 'risk matrix' of various traits and developed a risk assessment model in which the time taken for first detection of resistance was found to be a major determinant of resistance risk. In the absence of prior knowledge of resistance, such trait-based analysis can be used to predict resistance risk of new modes of action.

Based on practical experiences gained under diverse geographical situations over the years combined with observations drawn from pathological, biochemical and molecular investigations, examples of pathogens and fungicides with different levels of resistance risk are mentioned in Tables 2, 3, respectively.

Knowledge gained on likely risk of resistance development before a new fungicide is introduced for commercial use has proved quite helpful in developing effective disease control strategies in actual crop situations.

**Table 2** Examples of pathogens showing high, medium or low risk of resistance development

| Pathogen | Crop | Disease |
|---|---|---|
| High risk pathogens | | |
| *Botrytis cinerea* | Various | Grey mold |
| *Blumeris graminis* | Wheat, barley | Powdery mildew |
| *Corynespora cassiicola* | Soybean | Target spot |
| *Erysiphe necator* | Grapes | Powdey mildew |
| *Plasmopara viticola* | Grapes | Downy mildew |
| *Pseudoperonospora cubensis* | Cucurbits | Downy mildew |
| *Mycosphaerella) fijiensis* | Banana | Black sigatoka |
| *Pyricularia oryzae* | Rice | Rice blast |
| *Sphaerotheca fuliginea* | Cucurbits | Powdery mildew |
| *Venturia inaequalis* | Apple | Scab |
| Medium risk pathogens | | |
| *Albugo candida* | Brassica species | White rust |
| *Cercospora beticola* | Sugar beet | Leaf spot |
| *Colletotrichum acutatum* | Various | Anthracnose |
| *Penicillium digitatum* | Citrus fruit | Green mold |
| *Peronospora* spp. | Various | Downy mildews |
| *Phakopsora pachyrhizi* | Soybean | Asian rust |
| *Phytophthora capsica* | Various | Leaf blight, fruit rot |
| **Phytophthora infestans* | Potato, tomato | Late blight |
| *Pyrenophora teres* | Barley | Net blotch |
| *Venturia nashicola* | Asian pear | Scab |
| Low risk pathogens | | |
| *Cochliobolus miyabeanus* | Rice | Brown spot |
| *Hemileia vastatrix* | Coffee | Rust |
| *Phomopsis viticola* | Grapes | Cane and leaf spot |
| *Puccinia* spp. | Wheat, barley | Rusts |
| *Pythium* spp. | Various | Damping off |
| *Rhizoctonia* spp. | Various | Root rot. Foot rot |
| *Sclerotinia sclerotiorum* | Various | White mold |
| *Taphrina deformans* | Peaches, almonds | Leaf curl |
| *Tilletia* spp. | Cereals | Bunts |
| *Ustilago* spp. | Various | Smuts |

Source: Russell (2003), FRAC (2019)

*Reclassified from high risk to medium risk for all modes of action

## Fitness costs of resistance

After their emergence, knowledge of relative fitness of resistant strains in competition with sensitive strains is important in carrying out risk analysis as it helps to formulate fungicide use strategies to avoid or delay resistance build up. In order to determine fitness of resistant strains, competition experiments involving mixtures of resistant and sensitive isolates are conducted employing laboratory and glasshouse assays in which assessment of components such as mycelial growth rate, spore production potential, germination ability, spore dispersal, infection efficiency, etc. provide useful indication of the relative fitness of resistant strains. Traits such as reduced growth and sporulation and decreased pathogenic potential of resistant strain compared with sensitive strain are considered as fitness penalties or costs of resistance. As an example, when a mixture of azoxystrobin resistant and sensitive strains of *Magnaporthe oryzae* was inoculated on ryegrass, the resistant strain produced less conidia while sensitive strain increased in frequency indicating fitness penalty to resistance (Ma and Uddin 2009). Contrarily, no fitness costs were found with QoI resistance in *Plasmopara* viticola (Corio-Costet et al. 2010) and mefenoxam resistance in *Phytophthora erthroseptica* (Chapara et al. 2011).

However, it is quite cumbersome to measure fitness cost directly in pathogen populations as it involves bioassays with many isolates and is quite resource intensive. These days molecular diagnostic techniques offer cheaper options to study fitness costs. Genomic analysis is employed to better measure fitness costs of mutations encoding fungicide resistance (Hawkins and Fraaije 2018). The mutations conferring resistance may disrupt important physiological or biochemical processes thereby leading to lower fitness of the resistant strains. Hence, studies on the effect of point mutations on the activity of target enzymes are of great help in resistance risk analysis.

Fitness cost is influenced by environmental conditions under which the crop is growing. Several studies have shown that in the evolution of fungicide resistance, fitness penalties are influenced by environmental factors like temperature, nutrient status, and oxidative or osmotic stress, and variable results have been obtained depending

**Table 3** Resistance risk of major classes of fungicides

| Fungicide class | FRAC code | Target site | Resistance risk |
|---|---|---|---|
| Benzimidazoles<br>Thiophanates | 1 | ß-tubulin assembly in mitosis (cytoskeleton) | High |
| Phenylamides | 4 | RNA polymerase I (nucleic acids metabolism) | High |
| Quinone outside inhibitors (QoI) | 11 | Complex III: cytochrome bc1 -ubiquinol oxidase (respiration) | High |
| Dicarboximides | 2 | MAP/histidine-kinase (osmotic signal transduction) | Medium to high |
| Succinate dehydrogenate inhibitors (SDHI) | 7 | Complex II: succinate-dehydrogenase (respiration) | Medium to high |
| Oxysterol binding protein inhibitiors (OSBPI) | 49 | Lipid homeostasis and transfer (lipid transport) | Medium to high |
| Quinone outside inhibitor, stigmatellin binding (QoIS) | 45 | Complex III: cytochrome bc1 (ubiquinone reductase) at Qo site | Medium to high |
| Anilino-pyrimidines | 9 | Methionine biosynthesis (amino acids and protein synthesis) | Medium |
| Demethylation inhibitors (DMIs) | 3 | C14- demethylase (sterol biosynthesis in membranes) | Medium |
| Benzamides | 43 | Delocalisation of spectrin-like proteins (cytoskeleton) | Medium |
| Aza-naphthalenes | 13 | Signal transduction (unknown) | Medium |
| Phosphorothiolates | 6 | Methyltransferase (lipid synthesis) | Low to medium |
| Phenylpyrroles | 12 | MAP/histidine-kinase (osmotic signal transduction) | Low to medium |
| Carbamates | 28 | Cell membrane permeability (membrane function) | Low to medium |
| Carboxylic acid amides (CAA) | 40 | Cellulose synthase (cell wall biosynthesis) | Low to medium |
| Cyanoacetamideoximes | 27 | Unknown | Low to medium |
| Dithiocarbamates | M03 | Multi-site contact activity | Low |
| Copper compounds | M01 | Multi-site contact activity | Low |
| Chloronitriles | M05 | Multi-site contact activity | Low |
| Phthalimides | M04 | Multi-site contact activity | Low |

Adapted from FRAC Code List (2022) (www.frac.info)

on the factors studied (Hawkins and Fraaije 2018). DMI resistance in *Cercospora beticola* showed stability in the absence of flutriafol but resistance level was observed to decline after exposure to cold temperatures (Karaoglanidis and Thanassoulopoulos 2002). Similarly, azoxystrobin resistant *Phytophthora infestans* isolates showed increased level of sensitivity at elevated temperatures when exposed to a range of 13–25 °C (Lurwanu et al. 2020).

Stability of fungicide resistance is linked to fitness costs of resistant strains in competition with sensitive strains in a pathogen population. In the absence of fitness costs, the at-risk fungicide in a mixture continues selecting resistant strains eventually leading to poor efficacy, while with evident costs of resistance, the selection capability of the fungicide in a mixture is expected to decrease with least effect on disease control (Mikaberidze and McDonald 2015). Interestingly, in a study on QoI resistance (G143A) in grape powdery mildew pathogen *Erysiphe necator*, the stability of resistance was enhanced even after withdrawal of QoI fungicides indicating absence of fitness costs (Rallos et al. 2014).

## Resistance mechanisms

Resistance to fungicides is known to be conferred by various mechanisms including efflux of the fungicide or its reduced uptake, metabolic detoxification, alteration of target site that reduces binding of the fungicide, substitution of the target enzyme by synthesis of an alternative enzyme, and overproduction of the target protein. Additionally, some other mechanisms may also operate. Mechanisms of resistance development to DMIs, phenylamides and strobilurins have earlier been reviewed by Gisi et al. (2000). Alteration of the biochemical target site is the most common mechanism of resistance development to major groups of site-specufic fungicides including benzimidazoles, dicarboximides, DMI, QoI, CAA and SDHI fungicides. Mutations responsible for alteration of target sites leading to resistance development to these fungicide classes are mentioned in Table 1. Use of molecular biology tools has advanced our understanding of the mechanisms of fungicide resistance operating in phytopathogenic fungi. Knowledge of biochemical basis of resistance has led to

development of molecular techniques for rapid detection of resistant strains (Ma and Michailides 2005).

Phenylamide fungicides, known to inhibit polymerase I in rRNA biosynthesis in oomycetes, are the unique case. Despite being in use for more than 40 years, the exact mechanism of resistance to phenylamides is not fully understood. Inheritance of resistance studies have shown that phenylamide resistance is probably controlled by monogenic mechanisms involving one or two semi-dominant genes (Gisi and Sierotzki 2008). It is likely that multiple mechanisms may be involved. In a more recent study, analysis of the gene encoding RPA190 revealed that multiple mutations such as V1476G, P980S, and F382Y are responsible for metalaxyl resistance in *Phytophthora infestans* that can emerge in at least two independent pathways (Chen et al. 2018).

Fungi, like other microorganisms, have developed remarkable adaptive mechanisms over the course of their evolution. Using 'efflux pump' to resist a fungicide indicates that many fungi have developed molecular mechanisms for pumping the fungicide out (as they do with naturally occurring toxins) as it penetrates the cells.

## Multidrug resistance

Cross resistance of strains to other fungicides with same or similar modes of action is known for several fungicide groups such as triazoles, phenylamides, strobilurins and SDHI group fungicides. Nevertheless, ability of some pathogens to develop resistance to more than one unrelated fungicide in the field, known as multidrug resistance (MDR), has also been observed in many instances. The phenomenon of MDR is more common in human pathogens. The first case of MDR strains in a plant pathogen was observed in DMI resistant- *Penicillium digitatum* from citrus fruit which were shown to be simultaneously resistant to cycloheximide. *Botrytis cinerea*, causing grey mold in grapevine and other crops, represents the best example of MDR in plant pathogens. Under field conditions, *B. cinerea* is reported to develop multiple resistance to anilinopyrimidines, phenylpyrroles and hydroxyanilides, and three distinct phenotypes (MDR1, MDR2, MDR3) have been reported in vineyards in France and Germany (Kretschmer et al. 2009). All the three MDR phenotypes demonstrated enhanced fungicide efflux activity and there was an overexpression of efflux transporter genes. While activating mutations in the Mrr1 trascription factor, that controls the gene encoding ABC transporter AtrB, lead to emergence of MDR1 strains, a unique rearrangement in the promoter region of transporter gene *mfsM2* is responsible for MDR2 strains.

Similarly, MDR strains of *Mycosphaerella graminicola* from wheat grown in France and UK were identified with increased resistance against DMI fungicides as also with reduced sensitivity to boscalid, tolnaflate and terbinafine, having a frequency of 13% of the collected strains in France (Leroux and Walker 2011). The efflux transporter involved in this case has not been identified, though it is assumed that PDR5 may be associated. Recently in India, Ghule et al. (2020) have reported MDR strains of *Plasmopara viticola* showing resistance to both QoI and CAA fungicides confirming G143A mutation in *cytb* gene and G1105S mutation in *PvCesA3* gene, respectively. In majority of MDR cases known in plant pathogens, efflux pathway controlled by the gene encoding ABC transporter appears to be the most common mechanism.

## Role of FRAC

Fungicide Resistance Action Committee (FRAC), a pesticide industry-based body of CropLife International (formerly GIFAP), was formed in 1981 after experiences with benzimidazoles and phenylamides with regard to development of resistance in several target pathogens. The main purpose of FRAC is to develop guidelines for management of resistance so as to prolong the effectiveness of at-risk fungicides and thereby reduce crop losses. Members of FRAC are recognized experts from pesticide industry who are actively engaged in research on fungicide resistance. Several resource materials including monographs, resistance detection methods, grouping of fungicide modes of action (with codes) and resistance risk have been produced by FRAC that can be downloaded for consultation. These resources are of great help for the workers engaged in research on fungicide resistance.

FRAC has set up working groups and expert fora for different classes of at-risk fungicides. Working groups have been formed for fungicide classes viz. anilinopyrimidines (AP), sterol biosynthesis inhibitors (SBI), Quinone outside inhibitors (QoI), carboxylic acid amides (CAA), succinate dehydrogenase inhibitors (SDHI) and more recently introduced oxysterol binding protein inhibitors (OSBPI). Apart, there are three expert fora on benzimidazoles, dicarboximides and phenylamides. These working groups analyze resistance risk of respective fungicide classes in different pathogens and crops and develop common resistance management guidelines to avoid crop losses. These guidelines are revised from time to time and updated on FRAC website (www.frac.info) for the follow up. Apart from FRAC (International) headquartered at Brussels, there are regional FRAC groups in North America, Japan, Brazil and Argentina. In addition, there are associated fungicide resistance action groups (FRAG) in Australia, UK and few EU countries which are active at the local level.

## Resistance scenario in India

When compared to developed countries, overall use of fungicides is much lower in India. Majority of the fungicides used in India are broad-spectrum, multi-site, contact compounds such as inorganic sulphur, dithiocarbamates, copper compounds, phthalimeds, etc. Nevertheless, some of the site-specific, systemic fungicides like benzimidazoles, phenylamides, DMI, QoI, SDHI and CAA compounds are also in use against various diseases in fruits, vegetables, field and plantation crops.

Not much attention has been paid to investigate the problem of fungicide resistance in India, mainly because of general lack of awareness. Earlier studies on fungicide resistance were mainly confined to acquired resistance under laboratory conditions using mutagenesis or training of fungal pathogens on increasing fungicide concentrations, without looking into their practical implications under actual situations. However, during the last three decades, development of resistance to various site-specific fungicides has been reported in different plant pathogens under field conditions. Earlier, researchers have investigated practical cases of resistance to fungicides such as carbendazim (*Gloeosporium ampelophagum, Venturia inaequalis*), edifenphos *(Dreschlera oryzae, Pyricularia oryzae*), triadimefon (*Uncinula necator*), metalaxyl (*Plasmopara viticola, Phytopthora infestans*, *P. parasitica*, *Pseudoperonospora cubensis*) and few others. Characteristics of resistant strains, cross resistance to other fungicides, fitness potential and counter-measures have been worked out in some cases. Most of these cases have been reviewed by Thind (2012).

In the recent years, development of resistance in *Plasmopara viticola* to QoI (azoxystrobin, kresoxim methyl) and CAA (mandipropamid, dimethomorph) fungicides has been reported from vineyards in Maharashtra, and resistance was found to be conferred by G143A mutation in *cyt b* gene and G1105S mutation in *PvCesA3* gene, respectively (Sawant et al. 2017). MDR strains of *P. viticola* showing resistance to both QoI and CAA fungicides have also been reported in India (Ghule et al. 2020).

With the aim of developing guidelines for use of at-risk fungicides so as to avoid resistance build up in target pathogens and disseminate these to the stakeholders including field agencies and farmers, FRAC (India) chapter was formed in 1999 by CropLife, India. Among various activities, it published one technical bulletin on fungicide resistance scenario in India that contained information on prevailing resistance scenario in the country (Thind 2002). FRAC (India) is now a part of FRAC (Asia) chapter.

## Resistance management

With the knowledge gained from extensive research into the phenomenon of fungicide resistance both by the academia and industry experts, effective resistance management guidelines and strategies have been developed for different groups of fungicides. The basic principle of resistance management is to reduce selection pressure of the at-risk fungicide. Therefore, most resistance management strategies lay stress on limiting the applications of the single mode of action fungicide in a crop season and using it in alternation or mixture with a different mode of action fungicide. Other guidelines include starting fungicide application early in the season before disease progression accelerates (thus discouraging curative applications) and avoiding soil applications for foliar disease so as to avoid longer exposure to the pathogen. However, aspects such as using mixtures or alternations, higher or lower doses, and starting spraying early or waiting till a disease threshold is reached are long debated (Van Den Bosch et al. 2011).

The resistance management strategies aim not only at reducing selection for resistance but also at achieving optimal disease control and farmers need to be convinced to follow the strategies in practice (Corkley et al. 2022). Monitoring of pathogen populations and early detection of resistance are important in timely implementation of management strategies. Better understanding of the molecular mechanisms and genetic basis of resistance, that has allowed more rapid diagnosis of resistant strains, has proved quite helpful in resistance management.

## Using novel modes of action

It is needed to maintain a battery of different modes of action including conventional multi-site compounds for deployment in disease control as also resistance management strategies. Making use of advances in synthetic chemistry combined with biochemical and genetic approaches, fungicides with novel modes of action are introduced quite regularly that have helped much in managing resistance to earlier fungicides. It has been made necessary to determine resistance risk of a new compound and that it has no cross resistance to the existing modes of action. Somehow, environmental and toxicological regulations have slowed down the introduction of new fungicides. Recently, three fungicide groups, mainly specific to oomycete pathogens, viz. isoxazolines (oxathiapiprolin, fluoxapiprolin), triazolo-pyrimidines (ametoctradin) and tetrazolinones (metyltetraprole) have been introduced. The last two are QoI compounds but these do not show cross resistance to the existing QoI fungicides.

At present, 50 different modes of action are identified for various groups of fungicides.

## Role of conventional multisite inhibitors

Several conventional multisite, contact fungicides such as dithiocarbamates (mancozeb, zineb, propineb), phthalimides (captaf), and phthalonitriles (chlorothalonil) are still widely used to manage a wide range of diseases. Even after their extensive use for more than six decades, no cases of practical resistance to any of these protectant fungicides have been reported from anywhere. As these fungicides act at multiple sites in the fungal cells, it would require several mutations in target genes before resistance could emerge. With their multisite mode of action and broad-spectrum activity, these contact fungicides can serve as desirable components of mixtures with single-site, at-risk fungicides in resistance management strategies.

Among conventional multisite fungicides, dithiocarbamates have been the most favoured mixture partners. It was the role played by mancozeb, a member of ethylene-bis-dithiocarbamates, in delaying emergence of metalaxyl resistance in oomycete pathogens in the 1980s that set the tone for mixture strategy as a tool in resistance management (Thind and Hollomon 2018). Apart from delaying resistance build up, synergy has been observed between mancozeb and phenylamides which is an added advantage for better disease control. Using chlorothalonil as a partner fungicide in a mixture with QoI compound has been found to prolong the effectiveness of the latter to control Septoria leaf spot of winter wheat (Hobbelen et al. 2013). Such multiple-action fungicides are likely to serve as important resistance management tools and thus prolong the effective life of at-risk, site-specific fungicides in future as well.

## Mixtures vs. alternations

Use of at-risk fungicides in mixture or alternation with different modes of action including multi-site action fungicides is a common strategy to avoid or delay resistance development in target pathogens against site-specific fungicides. However, there are different points of view regarding their relative effectiveness. In general, mixtures of two or three fungicides, whether pre-packed or tank-mixes, are more commonly used. In majority of the cases, it has been demonstrated that with the use of mixtures there is slower evolution of resistance than with the use of rotations (Bosch et al. 2014). Mixtures have given better performance even when certain level of resistance is already there. Hence mixtures are considered a better option to prevent crop losses in the event of chance occurrence of resistance. However, in a recent study with *Zymoseptoria tritici* and fungicide mixtures of prothioconazole (DMI), benzovindiflupyr (SDHI) and carbendazim (benzimidazole) at minimal dose, Ballu et al. (2021) found that mixtures could select phenotypes with broad or multiple resistance due to selection pressure of individual components. They claimed that mixtures of single-site modes of action may not always be considered as an assured strategy for resistance management.

## Integrating non-chemical methods

Lately, in resistance management strategies, much emphasis is given on integration of fungicides with non-chemical methods of disease control. There is an increasing focus on the use of biologicals. Disease control measures involving integrated use of fungicides with moderately resistant crop varieties, biocontrol agents and suitable agronomic measures have proved quite helpful in reducing disease levels to get optimal yields. Since fungicides are used less frequently in an integrated disease management programme, pathogen populations experience reduced selection pressure that will lead to delay in emergence of resistant strains. Biocontrol agents are steadily becoming an integral component of disease management programmes.

## Use of resistance risk models

Some models have been developed that can predict development of resistance in practice. The models of fungicide resistance dynamics take into account the density of resistant and sensitive strains and derive predictions based on the effects of pathogen life cycle component and that of the fungicide use pattern on the evolution and spread of resistance (Van Den Bosch and Gilligan 2008). A resistance risk model using case history information on sequential emergence and evolution of resistant genotypes of four cereal pathogens (causing eye spot, Septoria blotch, powdery mildew and Fusarium ear blight) with reduced sensitivity to benzimidazoles, DMI, QoI and SDHI fungicides has been developed by Lucas et al. (2015).

Use of decision support systems (DSS), developed for several diseases based on disease epidemiological parameters, guide us to use fungicides based on disease risk. It has been seen that these DSS help to reduce frequency of fungicide applications by nearly 50 percent without compromising on disease control, and also limit the risk of resistance development.

## Conclusions

Fungicides are an important tool in our efforts to manage plant diseases. However, the performance of some fungicides, notably site-specific fungicides, is adversely impacted due to development of resistance in plant pathogens. Many of the fungicide groups including benzimidazoles, phenylamides, demethylation inhibitors, quinone outside inhibitors and lately succinate dehydrogenase inhibitors and carboxylic acid amides have experienced resistance problems with reduced levels of disease control in several instances. Nearly 50 years of research on fungicide resistance has advanced our understanding of the problem. Molecular biology has offered reliable diagnostic tools for rapid detection of resistant strains. Most of the resistance cases are linked to point mutations in target genes. The recently introduced unified system of labeling resistance-associated mutations will allow researchers to determine whether the changes in mutant amino acids were novel for a particular group of fungicides. Field-kit supported in situ monitoring can greatly help to understand pattern of resistance evolution in a pathogen population. Knowledge of fitness costs of resistance and risk assessment have proved useful in developing resistance management guidelines. However, combining fitness costs under realistic field situations remains a challenge.

Academia and industry have equally contributed in developing resistance management strategies. Majority of the strategies lay emphasis on reducing application frequency of at-risk fungicides and using them in mixture with other modes of action. Maintaining diversity in modes of action is important in resistance management. Though regulatory pressure has slowed down introduction of new fungicides, development of novel modes of action with lack of cross-resistance to the existing compounds remains a priority area in resistance management. Recently, three new groups of fungicides viz. isoxazolines, triazolo-pyrimidines and tetrazolinones, lacking cross resistance to the existing QoIs, have been introduced. Modeling of resistance dynamics and smartphone apps can be of much help in planning fungicide applications. There is an increased focus on the use of non-chemical means, particularly biocontrol agents, in disease control programmes so as to reduce selection pressure of fungicides. Nevertheless, the success of anti-resistance strategies depends much on the grower's willingness to implement the guidelines in practice.

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