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Phenylpyrroles: 30 Years, Two Molecules and (Nearly) No Resistance
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Phenylpyrroles are chemical analogs of the natural antifungal compound pyrrolnitrin. Fenpiclonil, but mainly fludioxonil are registered against multiple fungal crop diseases since over 25 years for seed or foliar treatment. They have severe physiological impacts on the pathogen, including membrane hyperpolarization, changes in carbon metabolism and the accumulation of metabolites leading to hyphal swelling and burst. The selection and characterization of mutants resistant to phenylpyrroles have revealed that these fungicides activate the fungal osmotic signal transduction pathway through their perception by a typical fungal hybrid histidine kinase (HHK). The HHK is prone to point mutations that confer fungicide resistance and affect its sensor domain, composed of tandem repeats of HAMP motifs. Fludioxonil resistant mutants have been selected in many fungal species under laboratory conditions. Generally they present severe impacts on fitness parameters. Since only few cases of field resistance specific to phenylpyrroles have been reported one may suspect that the fitness penalty of phenylpyrrole resistance is the reason for the lack of field resistance.

Keywords: fungicide, signal transduction, histidine kinase, resistance, fitness

THE ORIGIN OF PHENYLPYRROLES
Phenylpyrroles are chemical derivatives of pyrrolnitrin, a secondary metabolite produced by some bacteria from tryptophan (Floss et al., 1971). It was isolated for the first time from Pseudomonas pyrocinia in the 1960s (Arima et al., 1965) and showed strong antifungal activity against various animal and plant pathogenic fungi even under greenhouse conditions. Pyrrolnitrin or pyrrolnitrin producing Pseudomonas (e.g., P. fluorescens) proved phytoprotecting efficiency against Rhizoctonia solani, Alternaria sp., Fusarium sp., Verticillium dahliae, and Thielaviopsis basicola. Its activity was found stable for 30 days in the soil (Howell and Stipanovic, 1979) but sensitive to light decomposition. Consequently, two synthetic analogs have been successfully developed by Ciba-Geigy AG (now Syngenta AG) in the 1980s and introduced in the market for seed treatment and foliar use (reviewed in Leadbetter et al., 1994).

Fenpiclonil and fludioxonil are 3-cyano-4-phenylpyrrol analogs of pyrrolnitrin with largely increased photo-stability and similar antifungal activity (reviewed in Corran et al., 2008). These compounds differ by the substitutions at positions 2 and 3 of the phenyl ring (Figure 1). Fenpiclonil

Abbreviations: HAMP domain: domains conserved among histidine kinase, adenylate cyclase, methyl accepting proteins, phosphatases; HHK: hybrid histidine kinase; HK: histidine kinase; MAPK: mitogen activated kinase; PK-III: protein kinase III; ST: signal transduction.
(synthesized in 1984) was introduced in the market as seed-treatment in 1988 but rapidly superseded (1990) by the more stable and more active fludioxonil as foliar and seed-treatment (Leadbitter et al., 1994; Corran et al., 2008). To date, fludioxonil can be considered as major representative of the phenylpyrrole family of fungicides.

As non-systemic, surface fungicide, fludioxonil is registered for treatments at pre- and post-harvest stages on leaves, fruits and seeds. It has a principally prophylactic action against multiple fungal diseases provoked by ascomycetes or basidiomycetes. The list of crops registered for the use of fludioxonil and the associated pathogens (if known) is indicated in Table 1. Fludioxonil has no detectable activity on non-target organisms, such as baker’s yeast, men, plants, or animals (Gehmann et al., 1990).

Phenylpyroles inhibit all stages of fungal development, spore germination, germ-tube elongation, and mycelial growth (Leroux et al., 1992). The observed consequences are swollen hyphae with increased ramifications and apical lysis (Leroux, 1996) indicating that phenylpyroles might act on the intra-hyphal turgor and cell wall biosynthesis (Lew, 2010).

**EFFECT OF PHENYLPYRROLES ON TARGET FUNGI – MODE OF ACTION**

Jespers et al. (1994) observed extremely rapid intracellular accumulation of fenpiclonil in *Fusarium sulphureum* reaching its maximum in less than 1 min. Interestingly, the majority of the accumulated fenpiclonil can be washed off by water, suggesting that the phenylpyrrole penetrates the fungus through passive diffusion. The same study also showed that during the exposure of the mycelium to the phenylpyrrole. When the authors performed the same assay on crude mycelial extracts, they only observed a minor reduction of \[^{14}C\]-2-deoxyglucose phosphorylation under high concentrations of fenpiclonil, withdrawing the cytoplasmic hexokinase as sole or direct target of fenpiclonil (Jespers and De Waard, 1995).

Pillonel and Meyer tested the inhibition of protein kinase activities in *N. crassa* by phenylpyroles. They found that purified PK-III was inhibited by fenpiclonil and fludioxonil (Pillonel and Meyer, 1997). Although the concentration of phenylpyroles required for PK-III inhibition was found similar to that of rat PKC-inhibition, *N. crassa* PK-III does not seem to be neither a Ca\(^{2+}\)/calmodulin nor a cAMP regulated protein kinase (Judewicz et al., 1981; Ulloa et al., 1987). To some extend the inhibition of PK-III correlated with growth inhibition by fenpiclonil, but less by fludioxonil, raising the question if phenylpyroles, especially fenpiclonil, directly inhibit PK-III activity. Given the data of Pillonel and Meyer, this hypothesis has never been retained nor validated, since the concentrations required to inhibit the purified enzyme (IC\(_{50}\)) were much higher (up to 100 times in the case of fludioxonil) than those needed to inhibit fungal growth (EC\(_{50}\)). Either phenylpyroles do not inhibit PK-III by itself, acting rather indirectly, or they may affect different cellular targets.
### TABLE 1 | Crops and diseases registered for pre- or post-harvest treatment with phenylpyrroles.

| Crop | Pathogens controlled | Reference |
|------|----------------------|-----------|
| **Seed treatments and post-harvest uses** | | |
| Almonds | Coryneum beijerinckii, Monilinia spp. | Gehmann et al., 1990 |
| Avocado | Dothiorella iberica, Neofusisoccum australe, Neofusisoccum luteum, Neofusisoccum parvum, Phomopsis spp. | Gehmann et al., 1990 |
| Barley | Microdochium nivale, Fusarium spp., Ustilago hordei, Pyrenophora graminea, Cochliobolus sativus | Gehmann et al., 1990 |
| Beans | Rhizoctonia solani, Botrytis spp. | Gehmann et al., 1990 |
| Carrot | n.i | |
| Citrus fruit | Penicillum digitatum | Kanetis et al., 2007 |
| Cotton | Fusarium spp., Rhizoctonia solani, Thielaviopsis basicola | Gehmann et al., 1990, Corran et al., 2008 |
| Cucurbit vegetables | n.i | |
| Eggplant | Botrytis spp. | Corran et al., 2008 |
| Flax seed | n.i* | |
| Ginseng | n.i* | |
| Grapes | Botrytis cinerea, Glomerella cingulata | Gehmann et al., 1990 |
| Grass (forage, fodder, hay) | n.i* | |
| Jojoba | n.i* | |
| Kiwifruit | Botrytis cinerea | Brigati et al., 2009 |
| Lettuce | Sclerotinia minor | Gehmann et al., 1990 |
| Maize | Fusarium graminearum | Gehmann et al., 1990 |
| Tropical fruits | n.i* | |
| Peanut | Sclerotinia minor, Rhizoctonia solani | Gehmann et al., 1990, Corran et al., 2008 |
| Peas | Ascochyta spp., Fusarium spp., Peyronellaea pinodes | Gehmann et al., 1990, Corran et al., 2008 |
| Pineapple | n.i* | |
| Pistachio | Alternaria spp. | Ma et al., 2004 |
| Pome fruit | Penicillium spp., Botrytis cinerea | Errampalli, 2004; Zhao et al., 2010 |
| Pomegranate | Botrytis cinerea, Alternaria spp., Penicillium spp. | Palou et al., 2007; D’Aquino et al., 2010 |
| Potato | Fusarium spp., Helminthosporium solani, Boeremia exigua, Rhizoctonia solani, Alternaria solani | Gehmann et al., 1990, Gachango et al., 2012 |
| Rapeseed | Leptosphaeria maculans | Gehmann et al., 1990; Duan et al., 2013 |

### TABLE 1 | Continued

| Crop | Pathogens controlled | Reference |
|------|----------------------|-----------|
| Rice | Gibberella fujikuroi, Rhizoctonia solani, Gaeumannomyces graminis, Cochliobolus miyabeanus | Gehmann et al., 1990 |
| Rye | Microdochium nivale, Urocystis occulta, Monographella nivalis | Gehmann et al., 1990; Corran et al., 2008 |
| Sorghum | n.i* | |
| Soybean | Fusarium spp., Sclerotinia sclerotiorum, Rhizoctonia solani | Mueller et al., 1999; Corran et al., 2008 |
| Stone fruits (apricots, peaches, nectarines, cherries, plums) | n.i* | |
| Strawberry | Botrytis cinerea | Gehmann et al., 1990; Taguchi et al., 2012 |
| Sunflower | n.i* | |
| Sweet potato | Rhizopus stolonifer | Edmunds and Holmes, 2009 |
| Tomato | Botrytis spp., Alternaria solani | Gehmann et al., 1990 |
| Tropical fruits | n.i* | |
| Watercress | n.i* | |
| Wheat | Tilletia laevis, Microdochium nivale, Fusarium spp., Bipolaris sorokiniana, Phaeosphaeria nodorum, Monographella nivalis | Gehmann et al., 1990; Corran et al., 2008 |
| Terrestrial non-food uses | | |
| Turf | Rhizoctonia solani | Gehmann et al., 1990 |
| Ornamentals | Rhizoctonia solani | Gehmann et al., 1990 |

*n.i.: not indicated (crops without associated pathogens were extracted from the registration review of fludioxonil).

### FROM PHENYLPYRROLES TO OSMOTIC SIGNAL TRANSDUCTION

Glycerol accumulation is a consequence specific to the exposure to phenylpyrrole, dicarboximide and aromatic hydrocarbon fungicides. The selection of laboratory generated mutants resistant to the three categories of fungicides in *B. cinerea* correlated with osmosensitivity (Leroux et al., 1992; Faretra and Pollastro, 1993). Also *N. crassa* osmosensitive mutants os-1, os-2, os-4, and os-5 (Perkins et al., 1982) are resistant to dicarboximides, aromatic hydrocarbons, and phenylpyrroles (Fujimura et al., 2000; Zhang et al., 2002).
The corresponding genes and mutations have been cloned and identified in *N. crassa* and later in other fungi. The *os-1* gene encodes a class III HHK (Schumacher et al., 1997) whose mutations lead to fungicide resistance and osmosensitivity (Ochiai et al., 2001). The *os-2* gene on its turn encodes the osmosensing MAPK (Zhang et al., 2002), homologous to the MAPK of *Saccharomyces cerevisiae* involved in adaptation to high osmolarity name, high osmolarity glycerol, Hog1 (Hohmann, 2002). The fungicide resistant/osmosensitive phenotype of os-2 mutants is due to non-sense mutations. Finally, *os-5* and *os-4* are the MAPKK and MAPKKK encoding genes, respectively (Fujimura et al., 2003). The Os-5, Os-4, and Os-2 elements are equivalent to the yeast osmotic ST cascade. Altogether these items suggest that the phenylpyrroles (and dicarboximides) target the osmotic ST cascade, in particular the class III HHK Os-1.

An additional argument for this hypothesis is the fact that the yeast *S. cerevisiae*, devoid of this class of HHK, is insensitive to phenylpyrroles and dicarboximides. The introduction of a class III HHK, orthologous to Os-1, leads to sensitivity to phenylpyrroles, dicarboximides and aromatic hydrocarbons in *S. cerevisiae* (Motoyama et al., 2005). These results are in favor of the class III HHK as direct target of phenylpyrroles (and dicarboximides).

The possible mode of action is that fludioxonil, by binding to the class III HHK, mimics an osmotic stress through the activation of the Os-2/Hog1 MAPK (Figure 2A). This activation probably leads to multiple downstream reactions, such as activation of H+-ATPase, K+-influx and glycerol biosynthesis leading to increased intracellular turgor and membrane potential (Lew, 2010). Additional enzyme activities may be affected, e.g., hexokinase or sugar transporters (Jespers et al., 1994; Jespers and De Waard, 1995) that ultimately explain the phenotypes outlined above.

### RESISTANCE TO PHENYLPYRROLES

Until now only few cases of field resistance specific to fludioxonil have been reported; this despite the fact that for many fungal species (*N. crassa, B. cinerea, S. sclerotiorum, U. maydis, A. nidulans,...*) resistant strains could easily be obtained after mutagenesis and successive replication on fludioxonil supplemented medium (e.g., Avenot et al., 2005). These laboratory mutants display high resistance levels to phenylpyrroles, which is often associated with sensitivity to hyper-osmolarity and cross-resistance to dicarboximides and aromatic hydrocarbons (Ochiai et al., 2001; Leroux et al., 2002). In addition, most laboratory mutants, e.g., in *B. cinerea* and *A. brassicicola*, display developmental defects and reduced pathogenicity (Avenot et al., 2005; Ajouz et al., 2010; Ren et al., 2016). Adversely, no fitness penalty was found associated with dicarboximide resistance (and phenylpyrrole sensitivity) in field strains (Oshima et al., 2002, 2006). Notably, in some fungal species, no developmental defect besides osmosensitivity was found associated with phenylpyrrole resistance (Motoyama et al., 2005; Luo et al., 2012).

Field strains cross-resistant to phenylpyrroles and dicarboximides have been isolated from *A. brassicicola, A. longipes, and A. alternata* populations (Dry et al., 2004; Iacomi-Vasilescu et al., 2004; Avenot et al., 2005; Luo et al., 2012; Avenot and Michailides, 2015; Malandrakis et al., 2015). No significant developmental defects could be detected in the *A. brassicicola* resistant mutants and only moderate osmosensitivity (Avenot et al., 2005; Iacomi-Vasilescu et al., 2008). However, phenylpyrrole resistance seems limited in *Alternaria* field populations (Avenot and Michailides, 2015; Malandrakis et al., 2015) indicating a potential fitness penalty not detected under controlled laboratory conditions.

Recently, fludioxonil resistant strains have been isolated from *B. cinerea* field populations in China, at low levels (<3%). They present the typical osmosensitivity and developmental defects of fludioxonil resistant laboratory mutants (Ren et al., 2016) raising the question of their capacity to compete with sensitive and fitter strains and the selective pressure of fungicide treatments on these particular populations. Globally, specific resistance to fludioxonil does not exist among gray mold populations maintaining the high efficiency of this fungicide (Walker et al., 2013; Fillinger and Walker, 2016). However, multi-drug resistant (MDR) phenotypes due to increased fungicide efflux affect sensitivity to fludioxonil (Kretschmer et al., 2009). Although MDR does not reach resistance levels sufficient to alter field efficacy of fungicides at their registered field rates, the MDR1h phenotype of *B. cinerea* group S strains leads to the highest resistance levels to fludioxonil reported for field isolates (Leroch et al., 2013) – besides the specific resistance reported from China (Ren et al., 2016) – and impacts fludioxonil efficacy at least in in vitro assays (Rupp et al., 2016).

### FUNGAL HISTIDINE KINASES LINKED TO PHENYLPYRROLE RESISTANCE

As mentioned above, mutations conferring resistance to phenylpyrroles and dicarboximides map to class III HHKs, although one cannot exclude the presence of mutations in other components of the osmotic ST cascades that have not been specifically searched for. HHKs are ubiquitous, but typical fungal HHKs are absent from mammals and therefore constitute interesting targets for fungicide treatments. They are involved in cellular ST systems referred to as His-to-Asp phosphorelay. HHKs act as primary sensors for various environmental signals and initiate the adaptive response after autophosphorylation and subsequent phosphotransfer (reviewed by Bahn, 2008). Interestingly, the class III HHKs were shown to be cytoplasmic (Meena et al., 2010; Foureau et al., 2014), meaning that they sense fludioxonil intracellularly after its transmembrane diffusion.

Fungal HHKs are composed of the variable N-terminal sensor domain and the C-terminal domain, including the catalytic HK and ATPase domains that autophosphorylate the conserved
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FIGURE 2 | Signal transduction of phenylpyrrole perception in ascomycetes. (A) The fungicide signal is perceived by the HHK of class III, which transmits the signal to the osmotic MAPK cascade via the histidine-phosphate transfer protein (HPT) and the response regulator (RR). Phenylpyrrole treatment ultimately leads to MAPK-phosphorylation and activation of an adaptive response either through transcriptional activation in the nucleus or through the regulation of cytoplasmic proteins. The RR Skn7, under control of Ypd1, is also involved in the transcriptional regulation in response to phenylpyrrole treatment. The involvement of the HHK VI in the adaptation is less well documented. Protein names are those of S. cerevisiae, if different those of N. crassa are indicated in brackets. Full arrows indicate positive regulations, hashed arrows indicate either positive or negative regulations (different among fungal species), or direct interactions that remain to be demonstrated (reviewed in Bahn, 2008; Tanaka and Izumitsu, 2010; Jung et al., 2012; Herivaux et al., 2016). (B) Protein structure of class III fungal HHKs. The N-terminal domain, corresponding to the sensor domain, is constituted of 5–7 tandem repeats of HAMP motifs. The C-terminal half is composed of the catalytic domains HK, ATPase, and the RR. The conserved histidine residue in the HK domain is phosphorylated after hydrolysis of ATP by the ATPase. The phosphoryl group is then transferred to the conserved aspartate residue in the RR domain, which, ultimately, transfers the phosphoryl group to the HPT protein.

Histidine kinase, adenylate cyclase, methyl accepting proteins, phosphatases modules do not have strict sequence conservation, but a canonical coiled coil structure. HAMP subunits have...
two 16-residue amphiphilic helices (AS1, AS2) joined by a 14- to 15-residue connector segment. AS1 and AS2 have a seven-residue repeat pattern with hydroptic residues at the first and forth position, respectively (Parkinson, 2010). Rotation after signal perception is proposed to constitute the basic mechanism of HAMP mediated transmembrane signaling in bacteria (Airola et al., 2010, 2013; Klose et al., 2014).

The 5–7 repeats of HAMP modules and the cytoplasmic localization do not allow a simple transposition of the bacterial structure-function model to explain the mechanism of ST in fungal HKs. The number of repeat units varies across fungal species. Using S. cerevisiae as heterologous host the role of HAMP domains in ST has been investigated. In the case of Debaryomyces Hansenii class III HHK, HAMP deletion and yeast two hybrid studies led to the proposal of a functional model to explain the transduction of the hyperosmolarity or fludioxonil signal involving the five HAMP domains of the DhNik1 protein (Meena et al., 2010; Furukawa et al., 2012): The correct order of the HAMP domains is essential; HAMP1-3, 5 are essential for kinase activity, but HAMP4 is essential for the regulation of the HHK in response to a signal through its interaction with HAMP5. Using this approach, the authors showed that DhNik1 in the heterologous host S. cerevisiae has a functional kinase activity under standard conditions inhibiting the phosphorylation of the MAPK Hog1. Hyperosmolarity or fludioxonil inhibit DhNik1 activity leading to Hog1 activation. The interaction between two HAMP domains (HAMP4 and HAMP5) is essential for HHK inhibition. The authors also showed in the yeast model, that a constitutive active form of DhNIK1 confers resistance to fludioxonil. Among point mutations of N. crassa mutants displaying low resistance to fludioxonil (Ochiai et al., 2001), at least one of these mutations leads to a constitutive active form of the class III HK, conferring fludioxonil resistance to S. cerevisiae (Furukawa et al., 2012).

Mutations in fungal class III HHKs conferring resistance to phenylpyrroles and cross-resistance to dicarboximides generally induce phenotypes similar to deletion mutants (Viaud et al., 2006; Fillinger et al., 2012). They localize within or between the HAMP domains of the HHKs (Oshima et al., 2002; Alberoni et al., 2010; Fillinger et al., 2012; Firoz et al., 2015), while others are frameshift or non-sense mutations (Ochiai et al., 2001; Iacomi-Vasilescu et al., 2004; Duan et al., 2014; Ren et al., 2016). Altogether these results are in agreement with the hypothesis that in most cases loss-of-function mutations are responsible for fludioxonil resistance in plant pathogenic fungi (mainly laboratory mutants; reviewed in Defosse et al., 2015), but mutations leading to modified function or even constitutively active HHK may exist as well, probably at very low frequencies. Due to its essential role in many biological processes including pathogenicity (Viaud et al., 2006; Herivaux et al., 2016), loosing a class III HHK might explain the absence of fludioxonil field resistance in most plant pathogenic fungi.

CONCLUSION

Thirty years after their introduction in the fungicide market, the large spectrum phenylpyrroles still hide some mysteries. Although all characterized resistance mutations have been mapped to class III HHK genes, the corresponding protein has never been demonstrated as phenylpyrrole target. It has been shown that fludioxonil, the nearly unique representative of this class of fungicides, activates the osmosensing MAPK in divers fungi (Kojima et al., 2004; Yoshimi et al., 2005; Bahn et al., 2006; Hagiwara et al., 2007; Segmuller et al., 2007) which may be its real mode of action. One may hypothesize that this permanent stimulation of the hyper-osmolarity response via MAPK activation induces the observed pleiotropic phenotypes and, consequently, fungal death.

Another mystery is the absence (or low abundance) of fludioxonil field resistance. To our knowledge field isolates displaying specific resistant to fludioxonil have been detected only in Alternaria sp. (Iacomi-Vasilescu et al., 2004) and, very recently in B. cinerea (Ren et al., 2016). In most cases fludioxonil resistance due to mutations in the HHK gene seems to induce a strong fitness penalty; e.g., extremely reduced sporulation, osmosensitivity, loss of pathogenicity, etc. (Ziogas et al., 2005; Viaud et al., 2006; Ajouz et al., 2011; Malandrakis et al., 2015), definitely counter-selecting fludioxonil resistance. In the case of A. brassicicola, the absence of evident developmental defects in some fludioxonil resistant field isolates (Iacomi-Vasilescu et al., 2004), might be due to compensatory mutations in a given genetic background. Nevertheless spreading of these strains might be limited under field conditions due to some yet undetected defect. Therefore it might be suspected that evolution of fludioxonil resistance in fungal populations is strongly limited, unless additional mutations compensating the fitness penalty may arise and be selected. After 30 years of phenylpyrroles the chances to select such multiple mutations seem limited; otherwise they would have already appeared.

Another question raised while writing this review is the absence of alternative structural analogs of pyrrolnitrin that could have been produced by the chemical companies; an astonishing fact since fenpiclonil and fludioxonil have a large spectrum of activity, high efficiency and are not really facing resistance problems. Were similar components synthesized, but did not show comparable efficiency or stability? Are there problems with other phenylpyrroles that fenpiclonil or fludioxonil do not face? Is synthesis too complicated or expensive? At Ciba Geigy, among the multiple analogs tested, fenpiclonil and fludioxonil were the only molecules with the required properties for efficient fungicides (Leadbitter et al., 1994; Pillonel and Meyer, 1997) and their registration, suggesting potential problems in synthesis, activity, stability, and/or toxicity issues of other analogs.

With increasing resistance problems against medical antifungal compounds, class III HHKs have been considered as potential drug targets also against human fungal pathogens (Bahn et al., 2005; Nemecek et al., 2006; Chapeland-Leclerc et al., 2007; Randhawa et al., 2016), especially since the target is specific of the pathogen. Phenylpyrroles could constitute the next generation of clinical antifungals, but for this sector, we are not
aware of any compound in clinical testing, although pyrrolnitrin served as lead structure for pharmaceutical research (e.g., Unio et al., 1969). The absence of clearly characterized molecular interaction between phenylpyrroles and class III HHKs may explain the absence of clinical analogs of phenylpyrroles or other inhibitors of these proteins. Inhibition studies of known protein kinases may help the identification of new antifungal molecules [e.g., in the model fungus N. crassa (Pillonel, 2005), the plant pathogenic fungus Ustilago maydis (Tueckmantel et al., 2011; Grutter et al., 2012), the human pathogenic fungi C. neoformans and C. albicans (Tsuda et al., 2011; Lee et al., 2015)], but phenylpyrrole-analogs do not figure among the tested molecules.

From a fundamental point of view, the activation of the osmotic ST pathway by phenylpyrroles also raises questions. Do phenylpyrroles share the same ST elements as an hyperosmorality treatment? If they bind to the class III HHK, what are the interacting domains? Do they differ from those recognizing hyperosmorality (or dicarboximides)? Is resistance to fludioxonil conferred to by HHK loss-of-function mutations only, or are some of the mutations dominant active forms? These last questions require a thorough analysis of the ST processes after perception of phenylpyrroles, which may ultimately help understanding their mode of action.

**AUTHOR CONTRIBUTIONS**

JK and SF designed the plan of the manuscript. JK wrote sections 1–4, SF wrote sections 5 and 6 and completed the review.

**REFERENCES**

Airola, M. V., Sukomen, N., Samanta, D., Bobrat, P. P., Freed, J. H., Watts, K. J., et al. (2013). HAMP domain conformers that propagate opposite signals in bacterial chemoreceptors. *PLoS Biol.* 11:e1001479. doi: 10.1371/journal.pbio.1001479

Airola, M. V., Watts, K. J., Bilwes, A. M., and Crane, B. R. (2010). Structure of concatenated HAMP domains provides a mechanism for signal transduction. *Structure* 18, 436–448. doi: 10.1016/j.str.2010.01.013

Ajouz, S., Bardin, M., Nicot, P. C., and El Maatoussi, M. (2011). Comparison of the development in planta of a pyrrolnitrin-resistant mutant of Botrytis cinerea and its sensitive wild-type parent isolate. *Eur. J. Plant Pathol.* 129, 31–42. doi: 10.1007/s10658-010-9638-5

Ajouz, S., Nicot, P. C., and Bardin, M. (2010). Adaptation to pyrrolnitrin in Botrytis cinerea and cost of resistance. *Plant Pathol.* 59, 556–566. doi: 10.1111/j.1365-3059.2009.02230.x

Alberoni, G., Collina, M., Lanen, C., Leroux, P., and Brunelli, A. (2010). Field characterization of mutations in the two-component histidine kinase gene of Alternaria brassicicola. *Fungal Genet. Biol.* 51, 171–184. doi: 10.1016/j.fgb.2009.08.006

Arvind, L., and Ponting, C. P. (1999). The cytoplasmic helical linker domain of receptor histidine kinase and methyl-accepting proteins is common to many prokaryotic signalling proteins. *FEMS Microbiol. Lett.* 176, 111–116. doi: 10.1111/j.1574-6968.1999.tb13650.x

Arima, K., Imanaka, H., Kousaka, M., Fukuda, A., and Tamura, G. (1965). Studies on pyrrolnitrin, a new antibiotic. I. Isolation and properties of pyrrolnitrin. *J. Antibiot.* (Tokyo) 18, 201–204.

Avenot, H. F., and Michailides, T. J. (2015). Detection of isolates of Alternaria alternata with multiple-resistance to fludioxonil, cyprodinil, bosalid and pyraclostrobin in California pistachio orchards. *Crop Prot.* 78, 214–221. doi: 10.1016/j.cropro.2015.09.012

Bahn, Y.-S. (2008). Master and commander in fungal pathogens: the two-component system and the HOG signaling pathway. *Eukaryot. Cell* 7, 2017–2036. doi: 10.1128/ec.00323-08

Bahn, Y. S., Kojima, K., Cox, G. M., and Heitman, J. (2005). Specialization of the HOG pathway and its impact on differentiation and virulence of Cryptococcus neoformans. *Mol. Cell. Biol.* 15, 2285–2300. doi: 10.1091/mcb.E04-11-0987

Bahn, Y. S., Kojima, K., Cox, G. M., and Heitman, J. (2006). A unique fungal two-component system regulates stress responses, drug sensitivity, sexual development, and virulence of Cryptococcus neoformans. *Mol. Biol. Cell* 17, 3122–3135. doi: 10.1091/mbc.E06-02-0113

Brigati, S., Gregori, R., Bianchi, P., and Serrati, L. (2009). “Fludioxonil: a new active substance for kiwifruit protection against post-harvest rots,” in *Proceedings of the IX Convegno Nazionale dell’Actinidia*, Viterbo-Latina, Italia, 6–8 Ottobre (Firenze: Società di Ortoloforfrutticoltura Italiana), 285–289.

Catlett, N. L., Yoder, O. C., and Turgeon, B. G. (2003). Whole-genome analysis of two-component signal transduction genes in fungal pathogens. *Eukaryot. Cell* 2, 1151–1161. doi: 10.1128/EC.2.6.1151-1161.2003

Chapeland-Leclerc, F., Paccallet, P., Rusprich-Robert, G., Reboutier, D., Chastin, C., and Papon, N. (2007). Differential involvement of histidine kinase receptors in pseudohyphal development, stress adaptation, and drug sensitivity of the opportunistic yeast Candida lusitaniae. *Eukaryot. Cell* 6, 1782–1794. doi: 10.1128/EC.00155-07

Corran, A., Knauf-Beiter, G., and Zeun, R. (2008). “Fungicides acting on signal transduction,” in *Modern Crop Protection Compounds*, eds W. Krämer and U. Schirmer (Weinheim: Wiley-VCH Verlag GmbH), 561–580.

D’Aquino, S., Palma, A., Schirra, M., Continella, A., Tribulato, E., and La Malfa, S. (2010). Influence of film wrapping and fludioxonil application on quality of pomegranate fruit. *Postharvest Biol. Technol.* 55, 121–128. doi: 10.1016/j.postharvbio.2009.08.006

Defosse, T. A., Sharma, A., Mondal, A. K., Dugé de Bernonville, T., Latgé, J.-P., Calderone, R., et al. (2015). Hybrid histidine kinases in pathogenic fungi. *Mol. Microbiol.* 95, 914–924. doi: 10.1111/mmi.12911

Dongo, A., Bataille-Simoneau, N., Campion, C., Guillemette, T., Hamon, B., Lacomari-Vasilescu, B., et al. (2009). The group III two-component histidine kinase of filamentous fungi is involved in the fungicidal activity of the bacterial polyketide ambruticin. *Appl. Environ. Microbiol.* 75, 127–134. doi: 10.1128/AEM.00993-08

Dreyer, I. B., Yuan, K. H., and Hutton, D. G. (2004). Dicarboximide resistance in field isolates of Alternaria alternata is mediated by a mutation in a two-component histidine kinase gene. *Fungal Genet. Biol.* 41, 102–108. doi: 10.1016/j.fgb.2003.09.002

Duan, Y., Ge, C., Liu, S., Chen, C., and Zhou, M. (2013). Effect of phenylpyrrolyl fungicide fludioxonil on morphological and physiological characteristics of Sclerotinia sclerotiorum. *Pestic. Biochem. Physiol.* 106, 61–67. doi: 10.1016/j.pestbp.2013.04.004

Duan, Y.-B., Ge, C.-Y., and Zhou, M.-G. (2014). Molecular and biochemical characterization of Sclerotinia sclerotiorum laboratory mutants resistant to dicarboximide and phenylpyrrole fungicides. *J. Pest Sci.* 87, 221–230. doi: 10.1007/s10340-013-0526-6

Edmunds, B. A., and Holmes, G. J. (2009). Evaluation of alternative decay control products for control of postharvest Rhizopus soft rot of sweetpotatoes. *Plant Health Prot.* (in press). doi: 10.1094/PHP-2009-0206-01-RS

Errampalli, D. (2004). Effect of fludioxonil on germination and growth of Penicillium expansum and decay in apple cvs. Empire and Gala. *Crop Prot.* 23, 811–817. doi: 10.1016/j.cropro.2003.12.010

Faretta, F., and Pollastro, S. (1993). Isolation, characterization and genetic analysis of laboratory mutants of Botrytis fuckeliana resistant to the phenylpyrrole fungicide CGA 173506. *Mycol. Res.* 97, 620–624. doi: 10.1016/s0953-7562(09)81187-8
Iacomi-Vasilescu, B., Bataille-Simoneau, N., Campion, C., Dongo, A., Laurent, E., Serandat, I., et al. (2008). Effect of null mutations in the AbNIK1 gene on saprophytic and parasitic fitness of Alternaria brassicicola isolates highly resistant to dicarboximide fungicides. Plant Pathol. 57, 937–947. doi: 10.1111/j.1365-3059.2008.01864.x

Imanaka, H., Kousaka, M., Tamura, G., and Arima, K. (1965). Studies on pyrochelin, a new antibiotic. J. Structure of pyrochelin. J. Antibiot. (Tokyo) 18, 207–210.

Jespers, A. B. K., Davidse, L. C., and De Waard, M. A. (1994). Interference of the phenylpyrrole fungicide fenpiclonil with membranes and membrane function. Pestic. Sci. 40, 133–140. doi: 10.1002/pes.1993.1014

Jespers, A. B. K., and De Waard, M. A. (1995). Effect of fenpiclonil on phosphorylation of glucose in Fusarium sulphureum. Pestic. Sci. 44, 167–175. doi: 10.1002/pe.780440210

Kloes, D., Voskoboinikova, N., Orban-Glass, I., Rickert, C., Engelhard, M., Klare, J. P., et al. (2014). Light-induced switching of HAMP domain conformation and dynamics revealed by time-resolved EPR spectroscopy. FEBS Lett. 588, 3970–3976. doi: 10.1016/j.febslet.2014.09.012

Kojima, K., Takano, Y., Yoshimi, A., Tanaka, C., Kikuchi, T., and Okuno, T. (2004). Fungicide activity through activation of a fungal signalling pathway. Mol. Microbiol. 53, 1785–1796. doi: 10.1111/j.1365-2958.2004.03424.x

Kretschmer, M., Leroch, M., Mosbach, A., Walker, A. S., Fillingier, S., Mernke, D., et al. (2009). Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus Botrytis cinerea. PLoS Pathog. 5:e1000696. doi: 10.1371/journal.ppat.1000696

Lavín, J. L., Ramírez, L., Ussery, D. W., Pisabarro, A. G., and Oguiza, J. A. (2010). Genomic analysis of two-component signal transduction proteins in basidiomycetes. J. Mol. Microbiol. Biotechnol. 18, 63–73. doi: 10.1159/000277694

Lee, W. J., Moon, J. S., Kim, S. I., Bahn, Y. S., Lee, H., Kang, T. H., et al. (2015). A phenylpropanoid glycoside as a calcineurin inhibitor isolated from Magnolia obovata Thunb. J. Microbiol. Biotechnol. 25, 1429–1432. doi: 10.4104/jmb.1506.06031

Leroch, M., Plesken, C., Weber, R. W., Kauff, F., Scalliet, G., and Hahn, M. (2013). Gray mold populations in german strawberry fields are resistant to multiple fungicides and dominated by a novel clade closely related to Botrytis cinerea. Appl. Environ. Microbiol. 79, 159–167. doi: 10.1128/AEM.02655-12

Leroux, P. (1996). Recent developments in the mode of action of fungicides. Pestic. Sci. 47, 191–197. doi: 10.1002/slet.1096-9063(199606)47:2<251::AID-PIS1455-3.3.CO;2-9

Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., et al. (2002). Mechanisms of resistance to fungicides in field strains of Botrytis cinerea. Pest Manag. Sci. 58, 876–888. doi: 10.1002/ps.566

Leroux, P., Lanen, C., and Fritz, R. (1992). Similarities in the antifungal activities of fenpiclonil, iprodione and tolclofos-methyl against Botrytis cinerea and Fusarium nivale. Pestic. Sci. 36, 255–261. doi: 10.1002/ps.2780360312

Lew, R. R. (2010). Turgor and net ion flux responses to activation of the osmotic MAPK kinase cascade by flavodoxin in the filamentous fungus
Neurospora crassa. *Fungal Genet. Biol.* 47, 721–726. doi: 10.1016/j.fgb.2010.05.007

Luo, Y. Y., Yang, J. K., Zhu, M. L., Liu, C. J., Li, H. Y., Lu, Z. B., et al. (2012). The group III two-component histidine kinase AHI1K is involved in fungicides resistance, osmosensitivity, spore production and impacts negatively pathogenicity in *Alternaria longipes*. *Curr. Microbiol.* 64, 449–456. doi: 10.1007/s00284-012-0939-8

Ma, Q., Roy, F., Herrmann, S., Taylor, B. L., and Johnson, M. S. (2004). The Aer protein of *Escherichia coli* forms a homodimer independent of the signaling domain and flavin adenosine dinucleotide binding. *J. Bacteriol.* 186, 7456–7459. doi: 10.1128/JB.186.21.7456–7459.2004

Malandrakis, A. A., Apostolidou, Z. A., Markoglou, A., and Flouri, F. (2015). *Alternaria longipes* fungicides resistance, osmosensitivity, spore production and impacts negatively in filamentous fungi. *Eukaryot. Cell* 14, 426–570.

Randhawa, A., Chawla, S., and Mondal, A. K. (2016). Functional dissection of HAMP domains in NIK1 ortholog from pathogenic yeast *Candida lusitaniae*. *Gene* 577, 251–257. doi: 10.1016/j.gene.2015.12.002

Ren, W., Shao, W., Han, X., Zhou, M., and Chen, C. (2016). Molecular and biochemical characterization of laboratory and field mutants of *Botrytis cinerea* resistant to fludioxonil. *Plant Dis.* 100, 1414–1423. doi: 10.1094/pdis-11-15-1290-re

Rupp, S., Weber, R. W., Detzel, P., Rieger, D., and Hahn, M. (2016). Spread of *Botrytis cinerea* strains with multiple fungicide resistance in German horticulture. *Front. Microbiol.* 7:2075. doi: 10.3389/fmicb.2016.02075

Schultz, J. E., Kanchan, K., and Zieglar, M. (2015). Intraprotein signal transduction by HAMP domains: a balancing act. *Int. J. Med. Microbiol.* 305, 243–251. doi: 10.1016/j.ijmm.2014.12.007

Chun, S. H., Park, B. H., and Yang, S. H. (2015). Molecular basis for the interaction between the Trichoderma polysporum pigmentation gene ps.1080 and the *Botrytis cinerea* pigmentation gene ps.302. *Pestic. Sci.* 99, 67–74. doi: 10.1002/ps.4316

Umio, S., Kariyone, K., Tanaka, K., and Nakamura, H. (1969). Total biochemical characterization of laboratory and field mutants of *Botrytis cinerea* resistant to fludioxonil. *Int. J. Med. Microbiol.* 305, 243–251.

Tsuda, K., Nishii, N., Umeyama, T., and Uehara, Y. (2011). Identification of *Neurospora crassa* BLD1 as a specific or baseline sensitivity to fludioxonil and pyrimethanil in *Botrytis cinerea* and *Neurospora crassa* plants with multiple resistance to single site inhibitors and mancozeb. *Phytopathology* 101, 549.

Viaud, M., Fillinger, S., Liu, W., Polepalli, J. S., Le Pecheur, P., Kunduru, A. R., et al. (2016). Identification of *Ustilago maydis* aura2 kinase as a novel antifungal target. *ACS Chem. Biol.* 6, 926–933. doi: 10.1021/acschembio.16c00112y

Twizeyimana, M., McDonald, V., Mayorquin, J. S., Wang, D. H., Na, F., Alkil, D. S., et al. (2013). Effect of fungicidal application on the management of avocado branch canker (Formerly *Dothiorella Canker*) in California. *Plant Dis.* 97, 897–902. doi: 10.1094/psid-06-12-0518-re

Ulio, R. M., Gilkin, G. C., Tellez-Inon, M. T., Torres, H. N., and Judewicz, N. D. (1987). A novel stimulator of protein phosphorylation in *Neurospora crassa*. *J. Bacteriol.* 169, 77–117. doi: 10.1128/JB.169.1.71–77.1987

Umio, S., Kariyone, K., Tanaka, K., and Nakamura, H. (1969). Total synthesis of pyrrotrinitin. I. synthesis of 3-arylpyrrole derivatives by knorr condensation. *Chem. Pharm. Bull.* 17, 559–566. doi: 10.1248/cpb.17.559

Viaud, M., Fillinger, S., Liu, W., Polepalli, J. S., Le Pecheur, P., Kunduru, A. R., et al. (2006). A class III histidine kinase acts as a novel virulence factor in *Botrytis cinerea*. *Mol. Plant Microbe Interact.* 19, 1042–1050. doi: 10.1094/mpmi-19-1042

Walker, A. S., Micoud, A., Rémucon, F., Grosman, J., Gredd, M., and Leroux, P. (2013). French vineyards provide information that opens ways for effective resistance management of *Botrytis cinerea* (grey mould). *Pest. Manag. Sci.* 69, 667–678. doi: 10.1002/ps.3506

Yoshimi, A., Kojima, K., Takano, Y., and Tanaka, C. (2005). Group III histidine kinase is a positive regulator of Hog1-type mitogen-activated protein kinase in filamentous fungi. *Eukaryot. Cell* 4, 1820–1828. doi: 10.1128/EC.4.11.1820–1828.2005

Zhang, Y., Lamm, R., Pillonel, C., Lam, S., and Xu, J. R. (2002). Osmoregulation and antifungal chemical treatments and controlled atmosphere storage to control postharvest antifungal treatments and controlled atmosphere storage to control gray mold and improve storability of ‘Wonderful’ pomegranates. *Postharvest Biol. Technol.* 43, 133–142. doi: 10.1016/j.postharvbio.2006.08.013

Parkinson, J. S. (2010). Signaling mechanisms of HAMP domains in chemoreceptors and sensor kinases. *Annu. Rev. Microbiol.* 64, 101–122. doi: 10.1146/annurev.micro.112408.134215

Perkins, D. D., Radford, A., Newmeyer, D., and Bjorkman, M. (1982). Chromosomal loci of *Neurospora crassa*. *Microbiol. Rev.* 46, 426–570.

Pillonel, C. (2005). Evaluation of phenylaminopirimidines as antifungal protein kinase inhibitors. *Pest. Manag. Sci.* 61, 1069–1076. doi: 10.1002/ps.1086

Pillonel, C., and Meyer, T. (1997). Effect of phenylpyrroles on glycerol accumulation and protein kinase activity of *Neurospora crassa*. *Pestic. Sci.* 49, 229–236. doi: 10.1002/psc(1096-9063(199703)49:3<5:229::aid-PSS5255>3.0.CO;2-T
populations from apple and pear in Washington state. *Postharvest Biol. Technol.* 56, 12–18. doi: 10.1016/j.postharvbio.2009.11.013

Zhou, Q., Ames, P., and Parkinson, J. S. (2009). Mutational analyses of HAMP helices suggest a dynamic bundle model of input-output signalling in chemoreceptors. *Mol. Microbiol.* 73, 801–814. doi: 10.1111/j.1365-2958.2009.06819.x

Ziogas, B. N., Markoglou, A. N., and Spyropoulou, V. (2005). Effect of phenylpyrrole-resistance mutations on ecological fitness of *Botrytis cinerea* and their genetical basis in *Ustilago maydis*. *Eur. J. Plant Pathol.* 113, 83–100. doi: 10.1007/s10658-005-1227-7

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