Title
Helping southern strawberry growers control gray mold in light of widespread fungicide resistance

Final Report

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Extension Proposal

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Objective
Determine resistance to 7 important classes of fungicides in *Botrytis cinerea* from commercial strawberry fields in Southern States, analyze the data, and provide farmers with timely, location-specific resistance management recommendations

Justification
Extensive fungicide sensitivity testing of populations from individual farms in North Carolina and South Carolina collected in 2011 demonstrated that resistance to fungicides is an acute problem in the southeastern United States (Fernández-Ortuño *et al.,* 2012, 2013; Grabke *et al.,* 2013, 2014) . Populations of *B. cinerea* from North and South Carolina revealed isolates with resistance to 6 of the 7 chemical classes used for gray mold control and resistance came in 15 different combinations or fungicide resistance phenotypes (Fernández-Ortuño *et al.,* 2014; Li et al., 2014). Locations tended to have a single dominant, location-specific resistance profile (LSRP) that consisted of resistance to multiple fungicides in fields sprayed weekly with site-specific fungicides. Although the most prevalent profile found in conventional strawberry fields consisted of resistance to FRAC 1, thiophanate-methyl, FRAC 11 pyraclostrobin, and FRAC 7 boscalid, differences in predominant profiles between strawberry fields were identified. Multifungicide resistance had accumulated over time by stepwise acquisition of single resistances (Li *et al.,* 2014) . The schematic in figure 1 shows that all resistant phenotypes likely derived from populations already resistant to FRAC 1 fungicides. For example, isolates resistant to FRAC 12 were likely to have derived from populations that were already resistant to FRAC 1, 7, 11 and to FRAC 1, 7, 11, and 9 (**Fig. 1**).

Multifungicide resistance in *B. cinerea* isolates is also well known in Florida, the largest strawberry production region on the USA east coast. In a study carried out between 2010 and 2012, 392 *B. cinerea* isolates were collected from Florida strawberry fields and evaluated for sensitivity to registered site-specific fungicides. The study documented widespread resistance to FRAC 7 boscalid, FRAC 11 pyraclostrobin, FRAC 12 fenhexamid, FRAC 9 cyprodinil, and FRAC 9 pyrimethanil (Amiri *et al*., 2013). Isolates resistant to two, three and four fungicides

from different chemical groups were also observed (Amiri *et al*., 2013). Reports from other states are rare or focus on just one chemical class.

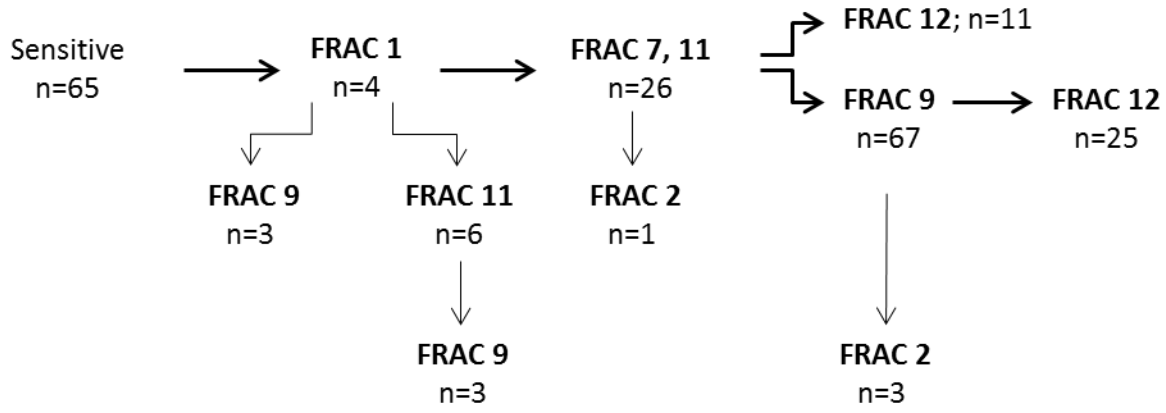

Figure 1. Accumulation (indicated by the arrows) of fungicide resistance phenotypes in 214 isolates from commercial strawberry fields of the Carolinas. Thicker arrows indicate accumulation of most commonly found fungicide resistant phenotypes. FRAC 1 = methyl benzimidazole carbamates, FRAC 11 = quinone outside inhibitors, FRAC 7 = succinate dehydrogenase inhibitors, FRAC 12 = hydroxyanilides, FRAC 9 = anilinopyrimidines, FRAC 2 = dicarboxamides, FRAC 12 = phenylpyrroles (low resistance). The number of isolates (n) of the accumulated resistance phenotype is indicated.

General resistance management recommendations, such as the rotation and/or mixture of registered chemical classes or the restriction of the number of applications per season delay the selection of fungicide resistance and thus extend the life span of site-specific fungicides. While these strategies are helpful to delay resistance in situations where the pathogen has not yet acquired resistance, some of them may fail in situations when multifungicide resistance is already observed. Let's use North and South Carolina strawberry fields as an example to illustrate how common strategies may backfire. As described above, resistance to FRAC 1, 7 and 11 was found to be among the most prevalent resistance profiles. The commonly used premixture of FRAC 7 and 11 fungicides would be expected to provide no protection from gray mold in populations with dual resistance because neither FRAC 7 nor FRAC 11 fungicides are effective. Furthermore a rotation between FRAC7/11 mixtures and FRAC1 fungicides would also be expected to provide very little to no control. In such locations the mixture or rotation of FRAC codes will also be an unnecessary expense for growers and pose unnecessary risk to the environment, worker, and consumer.

Because of widespread multifungicide resistance in the gray mold pathogen, it is critical that general resistance management strategies are supported by the identification of chemical classes with little to no resistance issues. Strawberry fields, even when in close proximity, may have a unique fungicide resistance profile (Fernández-Ortuño *et al.,* 2014) (**Fig. 2**) and therefore LSRPs need to be determined to avoid using ineffective chemical classes in standard rotations or mixtures. This may at first glance seem like an impossible task given the often large number of individual farms, the number of isolates and the different chemical classes that would need to be screened with traditional methods such as the determination of EC_{50} values for a large number of individuals per population. Molecular methods to detect resistance, although results may be

obtained more rapidly, do not appear suitable because multiple point mutations and even multiple mechanisms of resistance may occur for a single chemical class.

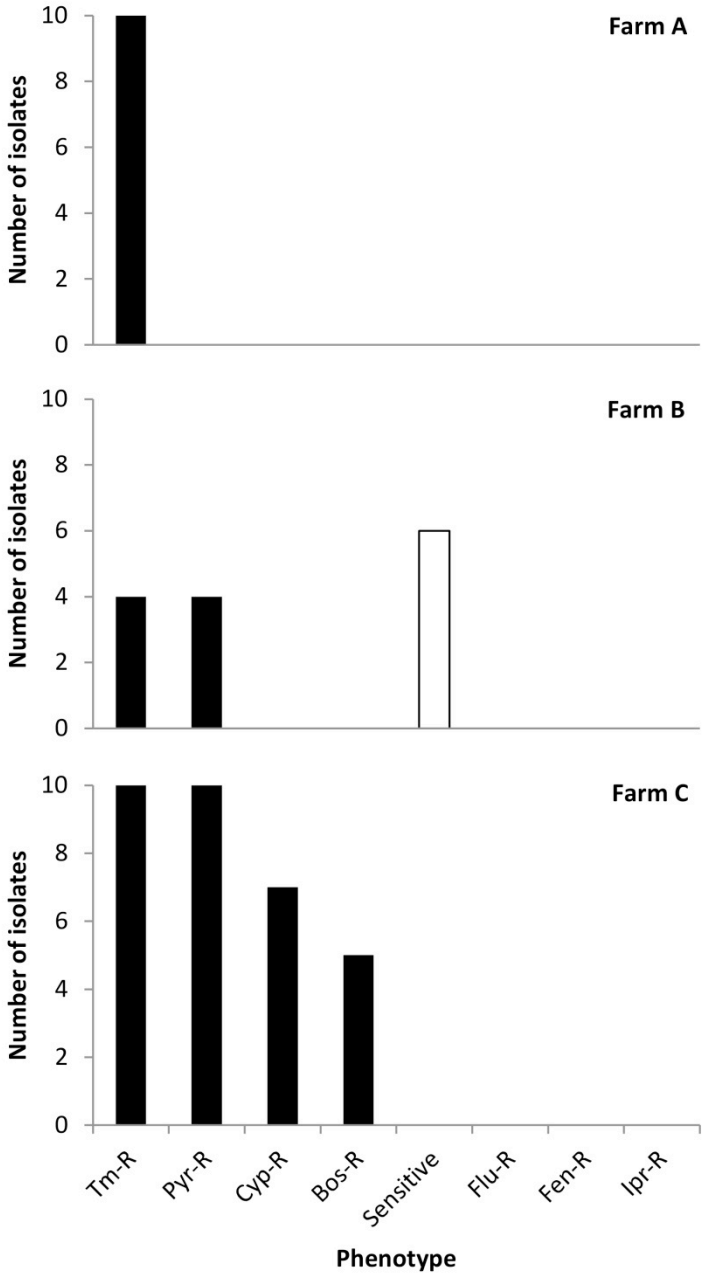

Figure 2. Fungicide resistance profile of *Botrytis cinerea* from strawberry fields of three farms located within 10 km of each other. Ten isolates were investigated for each location and some were resistant to multiple fungicides (farms B and C).

For example, resistance to FRAC 7 fungicides can be conferred by different mutations in the *sdh*B gene (Sierotzki and Scalliet 2013) and resistance to FRAC 2 fungicides is conferred by different mutations in the *Bos*1 gene and yet unknown mechanisms of resistance (Grabke *et al.,* 2014) . We developed an assay to screen for fungicide sensitivity in *B. cinerea* (Fernández-

Ortuño *et al.,* 2014) , which detects resistance regardless of the mechanism fast and reliably based on mycelia growth on fungicide-amended artificial growth media using a single discriminatory dose. This screening technique provides information of practical relevance.

Methodologies

Blossoms were sent to the Schnabel lab for analysis by growers, specialists and agents. To obtain conidia from blossom samples, a total of 20 to 40 strawberry blossoms with a degenerated, black torus (likely caused from freeze damage) were obtained from each strawberry field tested. After petals were removed, the blossoms were surfaced sterilized with 10% bleach for 1 min, rinsed with sterile water for 1 min, and allowed to air dry for 5 min. Then the blossoms were placed into 15 cm diameter Petri dishes containing sterile filter paper imbibed with 2 ml of sterile water. The blossoms were kept at 22°C for 2 to 4 days after which many became symptomatic for gray mold. During the first 24 hrs, the dishes were sealed with plastic bags to keep the relative humidity at 98 to 100%. For fruit samples, 10 to 12 individual strawberries with small (young) gray mold lesions were obtained from commercial fields; each fruit from a different plant with at least 5 buffer plants between sampled plants. Conidia were collected using individually wrapped sterile cotton swabs (Fisher Scientific, Pittsburgh, PA). The cotton tip was rubbed gently against the youngest area of sporulation (periphery of the lesion) of a fruit to capture conidia. The white cotton tip turned from pure white to lightly gray, indicating that sufficient conidia were collected; then, the swab was returned to its wrapper. Fungicide sensitivity of the bulk conidial isolates was defined using a novel mycelial growth assay.

A single dose for each fungicide was applied in mycelial growth assays to distinguish sensitive and resistant strains. The active ingredients, media and doses are listed in **table 1**. Inoculated plates were incubated at 22 ºC for 4 days and diametric colony growth was visually assessed in each well: (sensitive, S) for absence of growth, (low resistant, LR) for less than 20% diametrical growth, (medium resistant, MR) for less than 50% but more than 20% diametrical growth, and (resistant, R) for more than 50% diametrical growth compared to the control wells.

Table 1. Discriminatory concentrations and media used in spore germination and mycelial growth assays to monitor resistance in *Botrytis cinerea*

	Spore germination assay[y]		Mycelial growth assay	
Fungicides	**Concentration (µg/ml)**	**Medium[z]**	**Concentration (µg/ml)**	**Medium[z]**
Boscalid	1, 50	0.5% YEA	75	1% YBA
Cyprodinil	1, 25	0.5% SA	4	CzA
Fenhexamid	1, 50	1% MEA	50	1% MEA
Fludioxonil	0.1, 10	1% MEA	0.5	1% MEA
Iprodione	5, 50	1% MEA	10	1% MEA
Pyraclostrobin	n/d	n/d	10	1% MEA+100 µg/ml SHAM
Trifloxystrobin	0.1, 10	1% MEA+100 µg/ml SHAM	n/d	n/d
Thiophanate-methyl	1, 100	1% MEA	100	1% MEA

[y] Fungicide concentrations and media were previously described by Weber and Hahn (2011).
[z]CzA= CzapeK-Dox agar medium; MEA= Malt extract agar ; n/d= not determined; SA= Sucrose agar; SHAM= the alternative oxidase inhibitor salicyl hydroxamic acid; YEA= Yeast extract agar; and YBA= Yeast bacto acetate agar.

Resistance monitoring results were shared with growers and specialists in form of an official report detailing the resistance profile based on 10 strains examined to each chemical class together with resistance management recommendations.

Results

A total of 808 isolates were collected from southern states and 80 reports were sent out to growers with recommendations for disease control and resistance management. Results from 2014 indicate that thiophanate methyl resistance was stable despite the fact that none of our growers were using it in 2013 and 2014. Resistance frequencies to pyraclostrobin continue to be very high and was found in about 70% of the isolates. Compared to 2012, resistance frequencies to many other chemical classes, including FRAC 17 (fenhexamid), FRAC 12 (cyprodinil), and FRAC 2 (iprodione) increased. One surprising result was that resistance to FRAC 7 (boscalid) decreased to under 10% of the total population sampled. It is also noteworthy that although still rare, resistance to fludioxonil appears to increase.

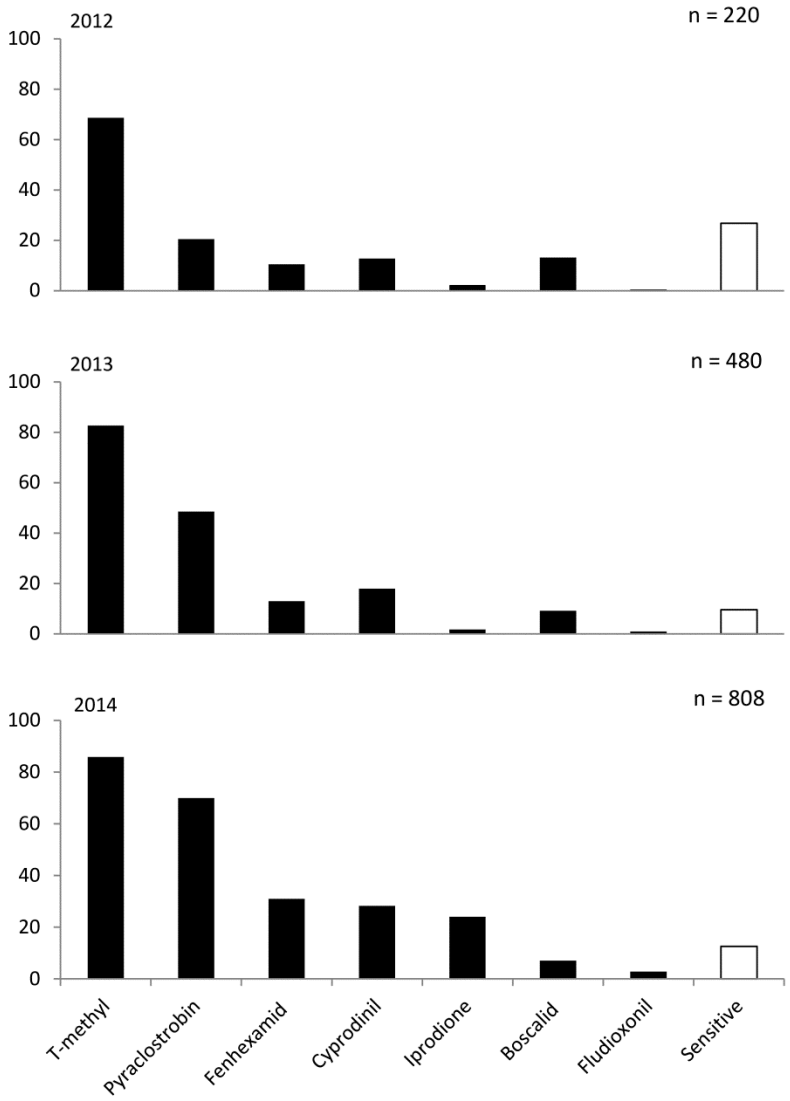

Fig. 3. Results of three years of resistance monitoring.

Conclusions

Our data show that fungicide resistance in *B. cinerea* is present in isolates from blossoms, indicating that resistance management needs to be implemented early in the season. The information provided through this monitoring program benefited all growers in that unnecessary sprays were avoided and gray mold was effectively controlled preharvest and postharvest. Growers are reporting improved disease control. The research provides further insights into the evolution and spread of fungicide resistance and will improve current anti-resistance management strategies.

Impact Statement

This resistance monitoring program serves all southeastern strawberry growers and provides critical, location-specific disease management recommendations. This service extends the life span of reduced risk fungicides, reduces pesticide input (through avoidance of ineffective sprays), catches resistance built up before an epidemic can cause preharvest and postharvest yield loss, makes producers aware about the need for resistance management, and teaches producers about novel resistance management options.

Citation(s) for any publications arising from the project

Grabke, A., D. Fernández-Ortuño, A. Amiri, X. Li, N. Peres, P. Smith and G. Schnabel 2014. Characterization of iprodione resistance in *Botrytis cinerea* from strawberry and blackberry. **Phytopathology**. 104:396-402.

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