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Dominant Selectable Markers for *Penicillium* spp. Transformation and Gene Function Studies

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Abstract

Penicillium spp. has been genetically manipulated and gene function studies have utilized single gene deletion strains for phenotypic analysis. Fungal transformation experiments have relied on hygromycin and hygromycin phosphotransferase (hph) as the main dominant selectable marker (DSM) system in *Penicillium* spp. This poses a limitation on the number of loci that can be analyzed and complemented in reverse genetic studies. Additionally, many economically important *Penicillium* spp. have not been evaluated to determine the utility of additional chemicals that can serve as DSMs. Therefore, six compounds were examined for 15 blue mold strains and their Minimum Inhibitory Concentrations (MICs) determined. Phleomycin, neomycin and G418 were deemed ineffective, as *Penicillium* spp. growth was observed on media amended with 1000 µg/ml of each compound. The efficacy of bialophos to inhibit fungal growth was intermediate, with MICs ranging from 250 to 1000 µg/ml and was species-dependent. However, chlorimuron ethyl and benlate had the lowest MIC values and minimal variation in efficacy within and between species. Therefore, benlate and chlorimuron ethyl are good candidates for use as since corresponding fungal resistance genes have been cloned, characterized and are available from a variety of public and academic sources.

Keywords: *Penicillium* spp.; Blue mold; Fungal transformation; Dominant selectable markers

Introduction

Blue mold, caused by *Penicillium expansum* and other *Penicillium* spp. is the most common and economically significant postharvest disease of apples and pears worldwide and losses have been estimated to exceed 4.4 million dollars annually [1-3]. In addition to *P. expansum, P. carneum, P. paneum, P. crustosum* and *P. solitum* have been isolated from apples with blue mold symptoms from the major tree fruit producing regions in Washington State and Pennsylvania [4,5]. Blue mold is characterized by a soft, watery rot that is light brown with blue-green colored conidia, often forming coremia, that develop on the fruit surface following advanced decay. *P. expansum* and other *Penicillium* spp. generally enter through wounds caused by stem punctures, bruises, and fingernail scratches that occur during harvest, but can also infect via natural openings (i.e., cracked lenticels, stem ends and open calyx). *Penicillium* spp. is troublesome because they reduce the availability of fresh fruit for consumption, contribute to food waste and produce mycotoxins (i.e., patulin, citrinin and penicillic acid) that contaminate processed fruit products [6]. Patulin is carcinogenic and is of primary concern in the United States and in Europe where strict limits on its amount in fruit juices and processed pome fruit products are set to a maximum level of 50 µg/L [6,7].

There is no source of host resistance in commercial apple cultivars as they are all susceptible to blue mold [8]. However, pioneering studies involving the identification and characterization of wild apple germplasm with resistance to blue mold have revealed multiple mechanisms associated with host resistance against *Penicillium* spp. infection [9-12]. It is envisioned that these findings will be utilized to incorporate blue mold resistance from specific wild apple accessions into existing commercial apple cultivars (e.g. 'Gala', 'Fuji', 'Honeycrisp', etc.). Current blue mold management options involve the use of postharvest fungicides, biological control formulations (BioSave™) and sanitation of bins and storage buildings [13,14]. Although, fungicide-resistant populations of *Penicillium* spp. have emerged in the packinghouse environment, thus greatly reducing the efficacy of fungicidal controls [15,16]. Hence, the lack of host resistance in cultivated apples, coupled with reduced efficacy of chemical controls, has demonstrated the need to develop targeted methods to manage blue mold decay which are facilitated by functional genetics studies in the fungus.

The first public release of an assembled and annotated *P. expansum* genome was published recently and has been available at the National Center for Biotechnology Information [17,18]. This has stimulated much interest in identifying genes and gene products that mediate pathogen virulence, spore germination and mycotoxin production [19-21]. Additionally, an *Agrobacterium tumefaciens* Mediated Transformation (ATMT) system for *P. expansum* using hygromycin and the hygromycin phosphotransferase (hph) gene as a single dominant selectable marker (DSM) has allowed researchers to analyze gene function, which has been developed [22]. Therefore, both functional genetic tools and corresponding genomic platforms have enabled researchers to utilize ATMT coupled with a single DSM to analyze *Penicillium* spp. gene function [19-21]. However, the use of

one selectable marker is not optimal or desirable to conduct molecular genetics investigations. Several situations arise when additional dominant selectable markers are needed for example: complementation of a mutant strain with a different dominant selectable marker, when several pathway enzyme mutants (e.g. patulin) are transformed in the same strain, and where dominant markers allow selection in prototrophic strains [23,24]. Therefore, the specific objective of this research was to screen multiple commercially available compounds suitable for *Penicillium* spp. selection, determine their MICs and evaluate their efficacy on different isolates of the same species to ensure broad functionality in *Penicillium* spp. causing blue mold decay of pome fruit.

Materials and Methods

Penicillium spp. isolation, culturing and storage

Nine of the *Penicillium* spp. isolates (F16, G2, G9, P24, R14, R19, SAH1, SAR1, SA4) examined were obtained from decayed apple fruit exhibiting blue mold symptoms from a commercial packing and storage facility located in Pennsylvania and a farm stand in Beltsville, Maryland by Dr. Wayne M Jurick II. Briefly, fruit surfaces were surface sanitized by spraying with 70% ethanol until run off and the asymptomatic tissue was removed from the lesion margin using aseptic techniques and a sterile scalpel. Fragments of apple tissue were placed on Petri plates containing Potato Dextrose Agar (PDA) and incubated at 25°C until fungal growth was evident. Pure cultures were obtained by touching the sporulating culture with a sterile loop and streaking out onto fresh PDA Petri plates. Monoconidial *Penicillium* spp. isolates were maintained on PDA plates and on PDA slants stored at 4°C. Additional isolates, F-Fr-J8, 16104, 42710 and Stanley A, were provided by Dr. Wojciech J Janisiewicz, USDA-ARS AFRS in Kearneysville WV. Isolate 3354 was provided by Dr. Richard Kim at Pace International in Wapato, Washington and RS1 was a gift from Dr. Robert A Spotts in Hood River, Oregon.

Morphological and genetic identification of *Penicillium* spp.

To determine the species-level identity of all 15 Penicillium isolates, two standard identification methods were utilized. Single spore cultures were preliminarily identified using morphological methods *in vitro* [25] (Figure 1). Confirmation of species-level identity was achieved using conventional PCR by sequencing ~700 bp of the β-tubulin locus as previously described [26]. Purified PCR products were subjected to Sanger sequencing using both forward and reverse primers. Geneious software was used to assemble a 2X consensus of each amplicon and was subjected to MegaBlast analysis (Table 1).

Isolate	Species	Cultivar	Year Isolated	Country/State	MegaBlast hit, % identity, E value
G2	*P. carneum*	Golden Delicious	2011	Pennsylvania	JF302650.1, 99, 0.0
R14	*P. crustosum*	Red Delicious	2011	Pennsylvania	JN112030.1, 99, 0.0
42710	*P. expansum*	Unknown	Unknown	Netherlands	KY426817.1, 100, 0.0
F-Fr-J-8	*P. expansum*	Unknown	1988	West Virginia	FJ012858.1, 100, 0.0
3354	*P. expansum*	Golden Delicious	2004	Washington State	FJ012853.1, 100, 0.0
F16	*P. expansum*	Fuji	2011	Pennsylvania	FJ012847.1, 100, 0.0
P24	*P. expansum*	Pink Lady	2011	Pennsylvania	FJ012853.1, 100, 0.0
R19	*P. expansum*	Red Delicious	2011	Pennsylvania	KY426817.1. 100, 0.0
SAH1	*P. expansum*	Honeycrisp	2011	Maryland	JN872743.1, 100, 0.0
SAR1	*P. expansum*	Red Delicious	2011	Maryland	FJ012858.1, 100, 0.0
SA4	*P. expansum*	Fuji	2010	Maryland	FJ012847.1, 100, 0.0
G9	*P. paneum*	Golden Delicious	2011	Pennsylvania	JF302651.1, 99, 0.0
16104	*P. solitum*	Unknown	Unknown	New York	FJ012875.1,99, 0.0
Stanley A	*P. solitum*	peach seed	2011	West Virginia	FJ012873.1, 99, 0.0
RS1	*P. solitum*	Unknown	Unknown	Oregon	JYNM01000623.1, 99, 0.0

Table 1: Penicillium species examined in this study. All isolates were propagated as monoconidial cultures, subsequently identified using standard morphological methods, and via sequencing the β-tubulin gene.

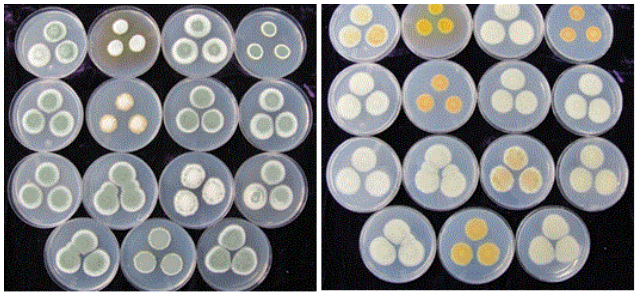

Figure 1: Photograph of the A) top and B) reverse of the 15 isolates analyzed in this study. From top to bottom, left to right *P. expansum* R19, *P. solitum* RS1, *P. expansum* SAR1, *P. solitum* Stanley A, *P. expansum* 3354, *P. solitum* 16104, *P. expansum* 42710, *P. carneum* G2, *P. paneum* G9, *P. expansum* F16, *P. expansum* F-Fr-J-8, *P. expansum* P24, *P. expansum* SA4, *P. crustosum* R14, and *P. expansum* SAH1. *Penicillium spp.* cultures were grown in a temperature controlled incubator at 25°C for 4 days and 25 µl of 1 × 10^4 conidia/ml suspension was inoculated as three spots on a PDA plate for each isolate.

Minimum inhibitory concentration (MIC)

MIC of six commercially available compounds for five different *Penicillium* spp. were conducted using conidial suspensions from seven-day-old *Penicillium* spp. cultures grown on PDA that were harvested with 1 ml of filter sterilized 0.05% Tween 20-treated water. Conidial suspensions were vortexed for ten seconds, quantified using a hemacytometer, and adjusted to 1 × 10^4 conidial/ml. Technical grade compounds of benlate, chlorimuron ethyl, bialophos, G148, neomycin and phleomycin D1 ranging from 0 to 1000 µg/ml were added to Richards defined medium containing sucrose as a sole carbon source pH 7.0 with 15 g/l phytagel agar (Table 2). Amended medium was added to 96-well plates and allowed to cool. Twenty five microliters of quantified conidial suspension from each isolate were pipetted into three independent wells of a 96 well plate, and placed in a temperature controlled incubator for 4 days at 25°C with natural light. Plates were examined using dissecting and compound microscopes for fungal growth. MIC was determined as the lowest concentration of compound that halted conidial germination and inhibited mycelial proliferation *in vitro*. Experiments were conducted twice using separate 96-well plates and different PDA plates for each isolate as the source of conidia. Additionally, the efficacy of benlate, chlorimuron ethyl, and bialophos on ten different *P. expansum* and *P. solitum* isolates was carried out using Richards defined medium in 96-well plates amended with 0, 1, 5 and 10 µg/ml benlate or chlorimuron ethyl and 0, 100, 250, 500, 750 and 1000 µg/ml bialophos (Table 3). The experiments were conducted as indicated above, MIC determined and was repeated.

Compound (µg/ml)							
Isolate	**Species**	**Benomyl**	**Bialophos**	**Chlorimuron ethyl**	**G418**	**Neomycin**	**Phleomycin D1**
G2	*P. carneum*	1	>1000	5	>1000	>1000	>1000
R14	*P. crustosum*	1	250	5	>1000	>1000	>1000
R19	*P. expansum*	1	250	5	>1000	>1000	>1000
G9	*P. paneum*	1	>1000	5	>1000	>1000	>1000
Stan A	*P. solitum*	1	250	5	>1000	>1000	>1000

Table 2: Minimum Inhibitory Concentration (MIC) of 6 compounds (µg/ml) for five monoconidial *Penicillium* spp. isolates that cause blue mold on apple fruit. MIC is defined as the concentration of chemical that halted conidial germination and prevented fungal growth four days post inoculation.

Results

Morphological and genetic identification of Penicillium species

Fifteen Penicillium isolates, representing four different species from six U.S. apple producing states and one from the Netherlands, were obtained from a variety of sources (Table 1). The majority were isolated from decayed apple fruit with blue mold symptoms from five commercial apple cultivars, one from peach seed, and four from unknown sources. All isolates were identified using morphological and molecular methods as described [25,26]. A ~700 bp portion of the β-tubulin amplicon was subjected to MegaBLAST analysis and, was found to be 99-100% identical with 0.0 E values, to cognate *Penicillium* spp. from published sources, previously deposited in Genbank. Thus, confirming the morphological determination of each species (Table 1).

Inhibition of Penicillium species growth *in vitro*

Minimum Inhibitory Concentration (MIC) was observed four days post inoculation for benlate, chlorimuron ethyl and bialophos at 1, 5 and 250 µg/ml respectively for *P. expansum, P. crustosum* and *P. solitum* (Table 2). *Penicillium carneum* and *P. paneum* had high levels of resistance to bialophos and grew on 1000 µg/ml but had MIC of 1 and 5 µg/ml for benlate and chlorimuron ethyl. All *Penicillium* spp. isolates had abundant growth on three compounds G418, neomycin and phleomycin D1 that developed vigorously growing fungal colonies at 1000 µg/ml for all five *Penicillium* spp. isolates.

Variation in MIC amongst *Penicillium expansum* and *P. solitum* isolates

To evaluate variation between isolates, several *P. expansum* and *P. solitum* isolates, were selected and tested against three compounds that were most effective to inhibit *Penicillium* spp. growth. Ten single spore *Penicillium* spp. isolates (8 *P. expansum* and 2 *P. solitum*) obtained from five different states (Maryland, New York, Oregon, Washington State, West Virginia and Pennsylvania,) including one international isolate (Netherlands), from five apple cultivars ('Fuji', 'Golden delicious', 'Honeycrisp', 'Pink Lady', and 'Red Delicious') isolated over multiple years from 1988-2011 were tested against three compounds (Table 2). Benlate, chlorimuron ethyl, and bialophos had MICs that were consistent between isolates. Benlate was the most inhibitory at 1 µg/ml for all isolates except for SA4 and RS1 (>10 µg/ml and 5 µg/ml respectively), inhibition by chlorimuron ethyl ranged from 5-10 µg/ml for all isolates, and bialophos was consistent at 250 µg/ml except for isolates F16 (750 µg/ml) and 16104 (500 µg/ml) (Table 3).

		Compound (µg/ml)		
Isolate	**Species**	**Benomyl**	**Bialophos**	**Chlorimuron ethyl**
42710	*P. expansum*	1	250	10
F-Fr-J-8	*P. expansum*	1	250	10
3354	*P. expansum*	1	250	5
F16	*P. expansum*	1	750	10
P24	*P. expansum*	1	250	10
SAH1	*P. expansum*	1	250	5
SAR1	*P. expansum*	1	250	10
SA4	*P. expansum*	>10	250	10
16104	*P. solitum*	1	500	5
RS1	*P. solitum*	5	250	10

Table 3: Minimum Inhibitory Concentration (MIC) of three compounds (µg/ml) for 8 monoconidial *Penicillium expansum* and 2 *P. solitum* isolates that cause blue mold on apple fruit. MIC is defined as the concentration of compound that halted conidial germination and prohibited fungal growth four days post inoculation.

Discussion

In this study, we conducted a screen of commercially available compounds to test against *Penicillium* spp. growth *in vitro* and to identify new materials that could be used as DSM's for *Penicillium* spp. transformation. We focused on chemicals that have been effective in transformation of other fungal species (*Ustilago maydis, Aspergillus flavus, Beauveria bassiana* and *Colletotrichum acutatum*) as DSMs with cloned corresponding fungal/bacterial resistance genes that function in different fungal pathogens [23,24,27-29]. We determined that G148, neomycin and phleomycin, were ineffective as fungal growth was observed for 5 different *Penicillium* spp. isolates on concentrations as high as 1000 µg/ml of each compound. This result was surprising for two of the three compounds (neomycin and phleomycin) which have similar modes of action to bleomycin that has been reported as an effective DSM for *P. chrysogenum* [30]. However, we found that benlate was excellent at inhibiting *Penicillium* spp. growth at low MIC values (~1 µg/ml) for the majority of *Penicillium* spp. isolates tested. This is not unexpected, since benlate is a member of the beta-tubulin inhibiting class of fungicides, in which thiabendazole (active ingredient in postharvest fungicide Mertect®) is routinely used in drenches and dips to control blue mold of apple [26]. It is hypothesized that previous exposure to TBZ, which is used as a postharvest decay control, may have contributed to higher levels (>1 ppm) of tolerance in one isolate of *P. expansum* and one of *P. solitum* obtained from decayed apple fruit. However, a higher MIC may also reflect natural variation in the level of resistance in the fungus which also may be present.

Benlate, also marketed as Benomyl® (DuPont), was used as a broad spectrum fungicide that inhibits the polymerization of β-tubulin monomers composing the cytoskeleton of the cell. Benlate, and corresponding β-tubulin resistance genes, have been successfully used as DSMs in other pathosystems for fungal transformation (i.e., *U. maydis*, *A. flavus* and *P. expansum*). Polyethylene glycol-mediated (PEG) transformation of *U. maydis* was accomplished using benlate and the Tub gene, which functioned just as well as hygromycin and thus expanded the molecular toolkit for this fungus [23]. Previous work [31] demonstrated that benomyl could function as a DSM in *P. expansum* via PEG-mediated transformation. However, results from their study showed low transformation efficiency and unstable transformants following selection on benlate-amended medium. Our results show that benlate can be used for a variety of *Penicillium* spp. and adds to the current body of knowledge as we defined a MIC for benlate based on 15 isolates from five different *Penicillium* spp. Our findings serve as a platform for transformation and selection of *Penicillium* spp. carrying a benlate resistance gene from *Aspergillus* spp. [24]. The next logical step would be to build a binary vector containing a ben resistance gene flanked by the *A. nidulans* Trp promoter and terminators for use in combination with ATMT to ensure higher transformation efficiency and stable transformation of *Penicillium* spp.

Chlorimuron ethyl is the active ingredient in the herbicide Classic® (DuPont) that inhibits acetolactate synthase mediating isoleucine and valine biosynthesis in plants. Interestingly, this compound is also inhibits fungal growth *in vitro* in *Colletotrichum acutatum* and *Beauvaria bassiana* and has been successfully used as a DSM [27,29]. PEG-mediated and ATMT systems have utilized the *sur* gene from *Magnaporthe grisea* which confers resistance to chlorimuron ethyl. PEG-mediated transformation has been used with this DSM, had acceptable transformation efficiency and stable transformation with little impact on cultural morphology and or virulence for *C. acutatum* [27]. The same system was used as a DSM but in an ATMT system to transform *B. bassiana* and achieved stable, high transformation efficiency [29]. Our studies are the first to show that this compound can also inhibit *Penicillium* spp. growth (5-10 µg/ml) and has established a guide for its use when transforming *Penicillium* spp. with a binary vector carrying the *sur* gene construct to screen *Penicillium* spp. transformants.

Bialophos is the active compound in the herbicide Ignite® (Bayer Crop Science) which interferes with glutamine synthesis in crops. Bialophos, along with corresponding bar resistance gene from *Streptomyces hyrgroscopicus*, has been used as a DSM in the transformation of *Sclerotinia sclerotiorum* and *Pleruotus ostreatus* [28,32]. It is possible that bialophos may function successfully as a DSM in *Penicillium* spp. However, functionality may be limited as this compound exhibited the greatest variability in inhibiting *Penicillium*

spp. growth *in vitro*. Based on our findings, we suggest that researchers use this compound with caution, and perform a series of MIC tests using their isolates of interest before undergoing fungal transformation with a bar construct in a given *Penicillium* spp. strain.

Conclusion

In summary, we were able to show effective inhibition of fungal growth for 15 different *Penicillium* spp. isolates from 5 different species using two different compounds which resistance genes have been cloned and characterized. These genes and corresponding constructs are available from the Fungal Genetics Stock Center located at Kansas State University and directly from researchers. The MIC values serve as a reference for others to evaluate these compounds to determine the precise concentration needed to screen putative *Penicillium* spp. transformants. In the future, we will couple our findings from this study with ATMT, which has been established [22]. The use of routine molecular methods (i.e., marker exchange/in fusion reaction) to swap the hph gene of the pPK2 binary vector [33] with the Ben-resistant gene from a closely related species like *A. flavus* [24] or the *sur* gene from *M. grisea* [27] would enable fungal transformation with both of these selectable markers. Construction of these vectors serves as the next logical step to transform various *Penicillium* spp. to determine frequency of integration, mitotic stability, and impact on cultural phenotype of Ben-resistant and Sur-resistant *Penicillium* spp. transformants. Additionally, one could also use the binary vector that was constructed for *B. bassiana* [29], to transform *Penicillium* spp. and select on chlorimuron-ethyl amended medium. We foresee that these new compounds, in combination with ATMT, will increase the molecular tool kit for *Penicillium* spp. that cause postharvest decay of pome fruit and facilitate future molecular genetics investigations to target multiple gene pathways involving fungal virulence, toxin production and fungicide resistance.

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