

Wild Yeast Strains as Prospective Candidates to Induce Resistance Against Potato Late Blight (*Phytophthora infestans*)

Lee A. Hadwiger · Haley McDonel · Dean Glawe

Published online: 14 March 2015
© The Potato Association of America 2015

Abstract The potential for crop destruction by *Phytophthora infestans*, the causal agent of the Late Blight of potato has not diminished since its impact led to the Irish potato famine. Potato production generally requires frequent applications of synthetic fungicides to hold this disease in check. As possible alternatives to fungicides, we investigated wild yeasts as biocontrol agents. Ten strains of wild yeasts isolated from vineyards within Washington State were assessed for the ability to reduce effects of *P. infestans* in potato leaf assays, and for suppression of growth of selected plant pathogenic fungi in agar culture. *Metschnikowia pulcherrima* (Mp), *Curibasidium pallidicorallinum* (Cp), and *Candida saitoana* (Cs) strains applied to potato leaves prior to inoculation with *P. infestans* reduced symptoms in a manner suggestive of induced immunity. A narrow concentration range of Mp most favorably suppressed late blight symptoms. These and other wild yeast strains were shown to induce phytoalexin production in a pea system developed for monitoring nonhost resistance. Further work toward developing successful agricultural application of this biocontrol agent will require verification of resistance-inducing benefits under field conditions.

Resumen El potencial de destrucción del cultivo por *Phytophthora infestans*, el agente causal del tizón tardío de la papa, no ha disminuido desde que su impacto condujo a la hambruna irlandesa. La producción de papa generalmente requiere de aplicaciones frecuentes de fungicidas sintéticos

para mantener a raya esta enfermedad. Como posibles alternativas a los fungicidas, investigamos levaduras silvestres como agentes de biocontrol. Se evaluaron diez cepas de levaduras silvestres aisladas de viñedos dentro del Estado de Washington para su habilidad de reducir los efectos de *P. infestans* en ensayos de hoja de papa, y para la supresión de crecimiento de hongos fitopatógenos selectos en cultivo en agar. Cepas de *Metschnikowia pulcherrima* (Mp), *Curibasidium pallidicorallinum* (Cp), y *Candida saitoana* (Cs) aplicadas a hojas de papa antes de la inoculación con *P. infestans* redujeron los síntomas en una manera que sugiere inducción de inmunidad. Una amplitud reducida en la concentración de Mp fue la que más suprimió favorablemente los síntomas del tizón tardío. Se mostró que estas y otras cepas de levaduras silvestres inducen la producción de fitoalexinas en un sistema de chícharo desarrollado para el monitoreo de la resistencia de no hospedante. Trabajo a futuro hacia el desarrollo de la aplicación agrícola exitosa de este agente de biocontrol requerirá verificación de los beneficios de la inducción de la resistencia bajo condiciones de campo.

Keywords Biological control · Induced immunity · Phytoalexin · Fungicide alternative · Organic

Introduction

The use of microbe-induced disease resistance in plants offers prospects for avoiding the use of direct-contact fungicides. This alternative approach requires using microbes capable of inducing defense response, without causing an accompanying negative effect on the plant or on the plant's commercial product. Yeasts are promising biological control agents. *Cryptococcus albidus* (Ca) provided a low level of control of white

L. A. Hadwiger (✉) · H. McDonel · D. Glawe
Department of Plant Pathology, Washington State University,
100 Dairy Road, Pullman, WA 99164, USA
e-mail: chitosan@wsu.edu

H. McDonel
e-mail: haley.mcdonel@email.wsu.edu

D. Glawe
e-mail: glawe@wsu.edu

mold, a bean disease caused by *Sclerotinia sclerotiorum* (Reeleder 2003). Commercial yeast, *Saccharomyces cerevisiae*, increased the survival of sugar beet plants affected by *Fusarium oxysporum* (Shalaby and Mohamed 2008). Five strains of antagonistic yeasts were inhibitory to the radial growth of *Colletotrichum gloeosporioides* a pathogen of Papaya (Hamizah et al. 2013). The mechanisms by which the yeasts suppress disease are varied, ranging from competition for nutrients to direct antagonism (Filonow 1998; Helbig 2002; Buck 2002). There are multiple reports of biocontrol via the use of yeasts. For example, the gene expression profile induced by the bio-control yeast *Cryptococcus laurentii* in cherry tomato fruit, assayed by microarray analysis, revealed activation of defense genes including PR genes (Jiang et al. 2009). Other enzymes, such as those metabolizing H₂O₂ are increased from antagonistic yeast in association with salicylic acid in sweet cherry fruit (Chan and Tian 2006). Microbe contact with a plant may lead to activation of many host responses including hormone-controlled pathways (Truman et al. 2007). Secondary responses such as systemic acquired resistance (SAR) reportedly protects against subsequent infections in plant tissues distal to the tissue directly challenged by microbial inoculation. SAR reportedly is mediated by salicylic acid (SA) signaling but initially may require jasmonate (JA) (Durrant and Dong 2004). *Saccharomyces cerevisiae* recently was reported to release a DNase capable of activating the defense response of nonhost resistance in peas (Hadwiger and Polashock 2013).

In addition to direct trials of protectants on a targeted plant, the capacity to signal defense responses, such as phytoalexin production, can be assessed. Consequently, it is possible to assess potential signaling by yeast strains in a separate model nonhost-disease-resistance-assay system (Hadwiger 2008). For example, the activation of plant defense termed “nonhost resistance” has been assessed both on the bases of phytoalexin accumulations and Pathogenesis-Related (PR) genes. These responses were activated with the commercial yeast (*Saccharomyces cerevisiae*) (Hadwiger and Polashock 2013). This elicitation was explained in part by the release of a DNase enzyme capable of entering plant cells to cause fragmentation of DNA within nuclear chromatin. Yeast cell walls also contain molecules, that when released, become elicitors of the plant defense response (Hahn and Albersheim 1978). Yeast cell walls in general contain β -glucans, mannoprotein, and chitin (Lipke and Ovalle 1998). Chitin is converted to the derivative chitosan by the enzyme chitin deacetylase. Although closely-related, by way of glucosamine polymerization being common to both polymers, the glucosamine of chitin is mostly N- acetylated. Chitosan polymers contain mostly deacetylated glucosamines, and thus are highly basic and water soluble. The mode of plant defense gene activation by chitin is proposed to be via a receptor protein in the cell membrane (Wan et al. 2008) and by chitosan, via a direct

affinity to DNA observed both in vitro and in vivo within the plant nucleus (Hadwiger and Beckman 1980).

The present report describes evaluation of wild yeasts, previously isolated from grapes in vineyards, (Bourret et al. 2013) as biological control agents. The cultures of interest are described in Table 1. *Phytophthora infestans*, (Mont.) deBary, the causal organism of potato late blight, continues to be a devastating potato pathogen. Furthermore, potato production relies on nearly weekly fungicide applications of position fungicides on leaf surfaces in advance of the arrival of incoming sporangia. Furthermore, potato production by organic growers is complicated because none of the organically-synthesized pesticides can be used; instead organic growers rely on copper-containing and other component materials that are not considered as synthetic. Consequently, use of innocuous microbes such as yeasts applied prior to *P. infestans* invasions could be beneficial to both conventional and organic growers if they elicit resistance of potato plants to this pathogen. Such an approach could be particularly useful if populations of the applied yeasts are enhanced by successful reproduction on plant surfaces, while concurrently activating a defense response, or strongly competing with the pathogen.

Materials and Methods

Fungal and Plant Materials

Strains of wild yeasts used in this study were isolated and characterized previously by Bourret et al. (2013) (Table 1). Russet Norkotah potatoes leaves were from plants grown in potting soil in a greenhouse with 12 h light at 22°. *Phytophthora infestans* was provided by Dennis Johnson (Washington State University). The bean pathogen, *Fusarium solani* f. sp. *phaseoli* (strain W-8)(ATCC no. 38135) was used to generate nonhost resistance responses in pea tissue.

Table 1 Wild yeast strains isolated from wine grapes *Vitis vinifera* L. grown in the Pacific Northwest (Bourret et al. 2013)

Strain #	Strain ID	Species
4	P45A002	<i>Candida saitoana</i>
19	P34A004	<i>Metschnikowia chrysoperlae</i> (1)
22	P01C004	<i>Metschnikowia pulcherrima</i> (1)
23	P01A016	<i>Metschnikowia pulcherrima</i> (2)
34	P44D001	<i>Rhodospiridium babjavea</i>
41	P34D001	<i>Rhodotorula</i> sp. (4) (<i>glutinis</i>)
45	P01A006	<i>Aureobasidium pullulans</i> var. <i>pullulans</i>
46	P45C001	<i>Curvibasidium pallidicorallinum</i>
47	P40D005	<i>Holtermanniella festucosa</i>
50	P42B001	<i>Wickherhamomyces anomalus</i>

F. solani f. sp. pisi (ATCC no. 38136) generated a susceptible response. All cell concentrations were assessed from multiple dilutions by direct counts, read in a coulter counter. The pea pod tissue was excised from the cultivar, “Lance”.

Plant Materials and Leaf Assay

Potato leaves were harvested from greenhouse-grown (under controlled conditions) potato plants and washed twice in distilled, filtered water. The leaves were excised from with a razor blade to minimize tissue damage and thus not induce the production of wound hormone. Leaves were assembled in a stock wash, randomly selected for uniformity and placed (three each) onto a plastic screen on top of damp paper towels all within a clear plastic (9×9 in.) incubation container. Treatments were held at 18 C and at 100 % humidity with 12 h low fluorescent light/12 h dark. A range of concentrations of yeast cell suspensions (in ~50 µl volume) were administered to the central region of each leaflet of 3 or more leaves with a camel hair brush. All test result analyses represent repeated trials (Mp =3; Cp =2 and Cs =1), each with extended concentration ranges, 21 individual leaflet readings/concentration tested, for determining the reported averages.

Phytophthora infestans sporangia (approximately 25/leaflet) were inoculated at a central site on each leaflet. Leaves were maintained 7 days or longer for disease assessment. A disease index, based on the “percent of the total area of each leaflet visibly infected”, was used to record symptoms. The long term viability of the yeast on the potato leaf surface at the 7 day period was verified by re-isolation of Mp on standard PDA medium or PDA medium plates with 0.5 mg/ml streptomycin sulfate. The identity of the re-isolated wild yeast strain was based on culture characteristic and microscopic examination.

Phytoalexin Production

Yeasts were evaluated for ability to elicit non-host resistance phytoalexin (pisatin) response using the pea-endocarp-tissue model system (Hadwiger 2008). Pea endocarp tissue (0.5 g) was treated with different concentrations of the yeast cells. Pisatin accumulation within 24 h was compared to that induced by *Fusarium solani f. sp. phaseoli* (Fsph) macroconidia (bean pathogen). Pisatin was extracted in 5 ml of hexanes. Hexane was volatilized away and pisatin was re-dissolved into 1 ml of 95 % ethanol and quantitated at 309_{nm} (Cruickshank and Perrin 1963). Pisatin values were derived both from duplicate experiments and the averages and ranges recorded for each treatment.

The wild-yeast elicitation of resistance in the pea tissue assay was also monitored directly as suppression of linear growth of a pathogenic fungus (*Fusarium solani f. sp. pisi*) (pea pathogen) using light microscopy. Yeast suspensions

were applied 20 min prior to *F. solani f. sp. pisi* and its mycelial growth was recorded at 24 and 60 h on endocarp surface layer sections treated with analine blue.

Yeast-Related Effect on the Growth of *F. solani* in Defined Liquid Culture

Fusarium solani macroconidia were pre-germinated in Vogel’s medium (Vogel 1956) and ~50 germinated spores were applied to the vials containing 200 µl of each yeast concentration used for phytoalexin induction and the subsequent growth of *F. solani* was monitored at 24 and 60 h periods.

DNA Digestion Agar Plate Assay

DNA (0.2 µg/ml)(calf thymus, Sigma) was incorporated into PDA agar just prior to solidification. The DNA was pre-sterilized in 95 % ethanol and dissolved in sterile water. DNA degradation around sites inoculated with yeast strains and *F. solani f. sp. phaseoli* (Fsph), after 3–7 days was detected by overlaying the plate with a 0.001 µg/ml ethidium bromide solution for 24 h and detecting digested and non-digested regions by photographing the agar under UV light.

Results

Preliminary Assessments of Biocontrol Microbes

The attributes that may enable a microbe to be a biological control agent are not immediately known. One early indicator can be detected when a prospective biocontrol agent is grown on PDA agar in the presence of a typical fungal pathogen, which in this screening was the bean pathogen, *Fusarium solani f. sp. phaseoli* (Fsph) and look for developing growth inhibition zones. Fsph was earlier reported (Hadwiger and Beckman 1980) to elicit a strong defense response in pea tissue against a true pea pathogen, *F. solani f. sp. pisi* (Fspi). The wild yeast *Metschnikowia pulcherrima* (str. PO1C004)(Mp) strain of those in Table 1, was initially selected for its suppression of *Botrytis cinerea*. Mp was marginally able to suppress the linear growth of Fsph (Fig. 1). However, the germination and growth of Fsph spores cultured in suspensions of Mp (data not shown) within complete liquid medium (Vogel 1956) were not inhibited until the concentration of Mp cells reached 1×10^8 cell/ml, indicating that the yeast Mp had a limited ability for direct antifungal action.

DNase Release Attribute

An additional attribute reported for fungi (Hadwiger and Polashock 2013) was the release of a DNase enzyme



Fig. 1 A three week old culture of the yeast *Metschnikowia pulcherrima* (Mp) (center) inoculated with three surrounding cultures of *Fusarium solani* f. sp. *phaseoli* (Fsph) on PDA media. The photo illustrates suppression of Fsph growth, adjacent to Mp

capability of inducing an immune reaction in a plant host. Therefore, DNase potential was evaluated for Mp in comparison with that of Fsph. The photo in Fig. 2 indicates that both Mp and the Fsph fungus from the pea tissue model system release an agent capable of degrading DNA when grown on DNA incorporated PDA agar.

Yeast strains exhibiting fungal suppressive activity in these initial tests were assessed further on excised potato leaves, and eventually on whole greenhouse grown plants. The prospects for the development of a commercial biological-control method are assisted both by these initial observations and the renewed interest in developing yeasts as commercial agents for biological control (El-Tarabily and Sivasithamparam 2006). Understandably, field conditions will introduce more variables that will require additional attention.



Fig. 2 DNA degradation by Mp strain 22 (center) and Fsph (flanking cultures) grown on DNA-incorporated PDA agar for 3 days. The PDA plates subsequently were stained with ethidium bromide to detect the undegraded DNA under UV_{260 nm} light. The dark halo near the culture edge indicates regions of degraded DNA

Metschnikowia pulcherrima (Mp)-Leaflet Assay

Potato leaves were treated with various concentrations of Mp 30 min prior to receiving (~50 sporangia in 10 μ l) of *Phytophthora infestans* applied to the center of each leaflet. Representative symptoms developing after 7 days are shown in Fig. 3. Although lesions continue to expand as incubation periods extend beyond 7 days, the percent of leaf infected readings were most distinctive at 7 days since these lesions eventually cause total deterioration of the complete leaf. The photo also indicates that the infected control leaf can have leaflets that escape symptoms although the same inoculum was administered on all leaflets. Taking into account this variation, the treatment concentrations considered meaningful were those in which all leaflets showed few or no symptoms. Yeast concentrations distant from the optimum both higher and lower cell concentrations, induced little or no resistant against *Phytophthora infestans* and thus suggest that any effective benefit of the yeast cell treatment depends on concentration-precise treatments.

Maximal protection against *Phytophthora infestans* was found in the treatment using *Metschnikowia pulcherrima* (Mp) cells in the range of 4.3×10^6 through 3.5×10^7 cells/ml (Table 2). However, there were concentrations within that range that exhibited less effectiveness, and some extremely high or low concentrations of the Mp cells resulted in symptom development exceeding that on the inoculated water-treated control tissue. In general, optimal concentrations induced protection lasting at least 10 days. Up to 10 days the excised potato leaves did not appear to senesce and thus readings within this period appeared reliable.

To determine if the protective effect was due to direct contact of *P. infestans* with Mp, and if the protection can be maintained over a 24 h period prior to *P. infestans* inoculation, Mp cells also were administered 24 h in advance of *P. infestans*. The 24 h delay did not affect the protection afforded by of Mp cell concentrations of 1.3×10^7 through 5.2×10^7 (Table 3). Surprisingly, the medium concentration 2.1×10^6 of Mp cells/ml resulted in infection percentages

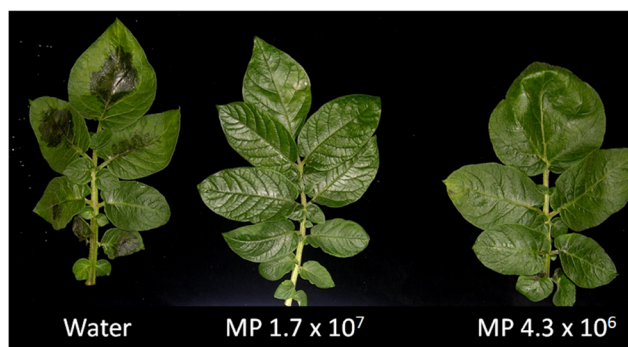


Fig. 3 The effect of *Metschnikowia pulcherrima* (Mp) cell numbers on *Phytophthora infestans* symptom expression on excised potato leaves

Table 2 Effect of *Metschnikowia pulcherrima* (MP) cells on Potato Late Blight symptoms

Treatment ^a		
MP Cells/ml	Leaflet inf. Ave % 7 Day reading	Leaflet inf. Ave % 10 Day reading
0	27.04	65.2
7×10^7	39.97	61.67
3.5×10^7	0	1.28
1.7×10^7	0.25	1.96
8.7×10^6	18.25	55.0
4.3×10^6	1.32	15.65
2.1×10^6	41.43	99.86

^a *Metschnikowia pulcherrima* (MP) cells (~50 µl volume) were administered to each leaflet (of 14 mature potato leaves) 5 min prior to the inoculation of ~50 *Phytophthora infestans* spores in 10 µl water. Leaves were held at 18 °C for 10 days in 100 % humidity

greater than that of the control. These results suggest that biocontrol of late blight using Mp cells would require strict adherence to optimal application rates. Viable yeast cells were re-isolated from the leaves after 7 days indicating an inherent benefit of biocontrol with yeasts may reside in an extended induction period when intact plants are utilized under field applications.

Assessment of Immunity Induction by Mp in Other Plant Systems

Mp cells also were assessed for ability to elicit phytoalexin production (Table 4), an immune response in peas. This experiment compared phytoalexin production induced by the yeast to that induced by the pathogen, *Fusarium solani* f. sp. *phaseoli* (Fsph). This fungus is a pathogen of bean that elicits

Table 3 Effect of *Metschnikowia pulcherrima* (Mp) cells 24 h prior to P. Infestans inoculation on Potato Late Blight symptoms

Treatment ^a		
MP Cells/ml	Leaflet inf. Ave % 6 Day reading	Leaflet inf. Ave % 10 Day reading
0	4.16	20.23
5.2×10^7	1.44	6.29
2.6×10^7	0.5	7.04
1.3×10^7	0.29	1.26
6.4×10^6	13.11	28.25
3.2×10^6	5.04	11.18
1.6×10^6	15.16	45.04

^a *Metschnikowia pulcherrima* (MP) cells (~50 µl volume) were administered to each leaflet (of 14 mature potato leaves) 24 h prior to the inoculation of ~50 *Phytophthora infestans* spores in 10 µl water. Leaves were held at 18 °C for 10 days in 100 % humidity

Table 4 Effect of 'Wild yeast fungus *Metschnikowia aff. pulcherrima* (Mp)(P01C004) and *Fusarium solani* f. sp. *phaseoli* (Fsph) spores on pisatin accumulation in pea endocarp tissue

Treatment ^a	Mp cells/ml ^b	Pisatin µg/g fr. wt..
Mp cells	5.1×10^8	38.9±0.1
Mp cells	2.5×10^8	29.3±2.1
Mp cells	1.2×10^8	12.3±12.3
Mp cells	6.4×10^7	22.3±2.9
Mp cells	3.2×10^7	7.3±3.3
Mp cells	1.6×10^7	2.0±2.0
Mp cells	8×10^6	7.4±1.5
Mp cells	4×10^6	3.8±3.1
Mp cells	2.0×10^6	6.7±4.1
Mp cells	1.0×10^6	2.4±0.5
Mp cells	5.0×10^5	9.7±5.3
Mp cells	2.5×10^5	3.4±3.4
Mp cells	1.2×10^5	8.8±2.2
Fsph spores	4×10^5	16.9±4.1
Fsph spores	8×10^6	74.3±6.9
Water	–	0.0±0.0

^a Pea endocarp tissue (pod halves, 0.5 g) was treated with 20 µl of the indicated treatments for 24 h. Pisatin was extracted with hexanes and quantified in 95 % ethanol at 309 nm

a nonhost resistance response in peas. Although small amounts of pisatin were induced by Mp concentrations as low as 1.2×10^5 cells per ml, the highest pisatin levels were found in treatments using $\sim 5 \times 10^8$ cells. The induction potential of some of the higher numbers of the small wild yeast-like cells appeared to match lower counts of *Fusarium* spores, possibly since the spores are much larger in size (Fig. 4). Spores of all of the yeast species isolates shown in Table 1 induced phytoalexin production (data not shown) when applied to the pea tissue at these high concentrations.

The potential of Mp cells to induce resistance against the true pea pathogen, *Fusarium solani* f. sp. *pisi* (Fspi), was further analyzed cytologically using the pea endocarp host tissue.

Mp yeast cell concentrations (strain 22) were applied to the surface cells of the pea endocarp tissue followed in 2 h with inoculations with true pea pathogen (*Fusarium solani* f. sp. *pisi*)(Fspi). Fig. 4 illustrates the complete suppression of Fspi 24 h following treatment with 5.1×10^8 Mp cells/ml. This response persisted for 72 h. The yeast cell concentrations assayed ranged from 2×10^9 through 1.6×10^7 /ml. However, growth of the Fspi pathogen was observed both at the highest concentration and at concentrations below 2.5×10^8 , again indicating the need to employ precise concentrations to obtain optimal resistance response. Both yeast spores and Fspi macroconidia visible on the endocarp surface appeared distorted within 24 h as a result of the plant response.

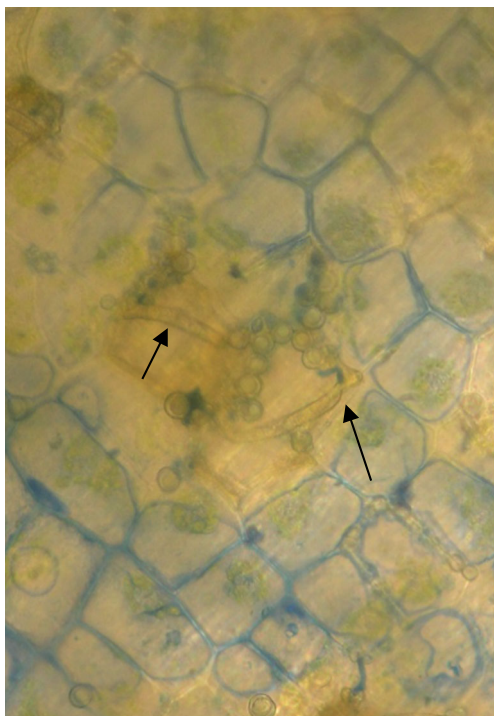


Fig. 4 The photo of *Fusarium solani* f. sp. *pisi* macroconidia (Fspi) indicates typical growth 24 h suppression following the administering of Mp cells to pea endocarp surface cells. Fspi was inoculated to the pea tissue 2 h following Mp. Arrows identify two distorted spores of Fspi. Adjacent Fspi spores experienced a ballooning of their walls. Mp cells are present but not clearly resolved due to their small size

Hypersensitivity coloration and nuclear distortion (following staining with the DNA specific stain, DAPI, and observed under UV light-not shown) were observed in the pea endocarp cells.

Two additional strains, *Curvibasidium pallidicorallinum* (Cp) and *Candida saitoana* (Cs), were selected for assessing protection-induction against *P. infestans* on potato leaves. All of the strains of Table 1 with similar cultural features and taxonomic similarities were thus tested (not shown) to determine if genera other than Mp were also able to induce pisatin accumulations. All strains induced pisatin.

Curvibasidium pallidicorallinum (Cp)-Induced Protection

The Cp-induced protection also occurred in the narrow cell concentration range of 5.1×10^6 through 2×10^7 /cells/ml with some deviation from this trend at the 1.0×10^7 cell/ml level (Table 5). This lack of complete linearity between the yeast spore concentration and the level of resistance induced, was also observed when Cp cell concentrations were applied to pea tissue to monitor pisatin production (Table 6). Thus there was a consistent potential for inducing immunity, however the variability due concentration likely indicates there are other auxiliary components, sequentially released from the yeast cells, that interfere in some undetermined suppressive manner.

Table 5 Effect of *Curvibasidium pallidicorallinum* (Cp) cells on Potato Late Blight symptoms

Treatment ^a		
No. of Cp cells/ml	Leaflet inf. Ave % 7 Day	Leaflet inf. Ave % 10 Day
0	59.5	85.07
4.0×10^7	42.61	58.67
2.0×10^7	9.19	10.39
1.0×10^7	37.28	52.23
5.1×10^6	21.23	31.89
2.6×10^6	64.31	75.44
1.3×10^6	57.83	64.28

^a *Curvibasidium pallidicorallinum* (Cp) cells (~50 μ l volume) were administered to each leaflet (of 14 mature potato leaves) just prior to the inoculation of ~15 *Phytophthora infestans* spores in 5 μ l water. Leaves were held at 18 °C for 10 days in 100 % humidity

The detection of hypersensitive coloration detectable in the pea tissue often accompanies optimum pisatin production.

Candida saitoana (Cs)– Induced Protection

Cs-induced protection appeared less effective than that from Mp or Cp. The maximum of protection by Cs was at the 5.1×10^7 cells/ml level (Table 7). Similarly, there was less induction potential evident in eliciting pisatin production (Table 8). This additional data indicated that not all of the wild yeast possess optimal abilities to induce plant immunity.

Table 6 Effect of yeast-like fungus *Curvibasidium pallidicorallinum* (Cp) (strain 46) on pisatin accumulation in pea endocarp tissue

Treatment ^a	Cp cells/ml	Pisatin μ g/g fr. wt.	H. S. color
Cp cells	4.8×10^7	109.9±39.5	++
Cp cells	2.4×10^7	107.0±11.7	++
Cp cells	1.2×10^7	64.9±6.9	+
Cp cells	6.4×10^6	19.9±1.3	nd
Cp cells	3.2×10^6	46.6±19.3	nd
Cp cells	1.6×10^6	28.8±1.5	nd
Cp cells	8×10^5	68.4±48.8	+
Cp cells	4×10^5	20.9±1.3	nd
Cp cells	2×10^5	12.8±2.0	nd
Fsph spores	2.8×10^6	249.3±20.2	++
Water	–	0.0±0.0	–

^a Pea endocarp tissue (pod halves, 0.2 g) was treated with 20 μ l of the indicated treatments for 24 h. Pisatin was extracted with hexanes and quantified in 95 % ethanol at 309 nm. Cp=*Curvibasidium pallidicorallinum*. nd not detected

Table 7 Effect of *Candida saitoana* (Cs) cells on Potato Late Blight symptoms

Treatment ^a		
Cs Cells/ml	Leaflet inf. Ave % 9 Day reading	Leaflet inf. Ave % 13 Day reading
0	3.13	33.76
5.1×10^7	0.57	10.5
2.6×10^7	2.78	24.17
1.3×10^7	6.79	45.59
6.4×10^6	2.69	15.53
3.2×10^6	4.75	14.57
1.6×10^6	3.72	15.36

^a *Candida saitoana* (Cs) cells (~50 µl volume) were administered to each leaflet (of 14 mature potato leaves) just prior to the inoculation of ~50 *Phytophthora infestans* spores in 10 µl water. Leaves were held at 18 °C for 13 days in 100 % humidity

Discussion

Previous decades of research identified multiple components released by microorganisms, including yeast-like fungi that exhibit capabilities for eliciting plant responses. The properties of these components have not been utilized to the fullest possible extent in the biological control of important plant diseases. The yeasts utilized in this study were evaluated for prospective use in commercial fields for managing Late blight of potatoes. Such bio-control options could be especially useful to organic growers unable to employ chemically-synthesized fungicides. The successful application of biological entities presents challenges well beyond those of direct-kill fungicides, as the biology of both the microbe and the plant tissue are involved, making their retention of effectiveness and viability in nature paramount.

Table 8 Effect of wild yeast fungus, *Candida saitoana* (Cs)(strain 4) on pisatin accumulation in pea endocarp tissue

Treatment ^a	CS cells/ml	Pisatin µg/g fr wt.
Cs cells	1.7×10^7	15.7±3.2
Cs cells	8.9×10^6	81.5±3.1
Cs cells	4.4×10^6	11.3±11.3
Cs cells	2.2×10^6	9.0±3.7
Cs cells	1.1×10^6	2.2±2.2
Cs cells	5.6×10^5	0.0
Cs cells	2.8×10^5	0.0
Cs cells	1.4×10^5	1.6±0.4
Cs cells	7.0×10^4	4.7±4.7
Fsph spores	7.5×10^6	180±13.0

^a Pea endocarp tissue (pod halves, 0.2 g) was treated with 20 µl of the indicated treatments for 24 h Pisatin was extracted with hexanes and quantified in 95 % ethanol at 309 nm

Yeasts isolated from nature have evolved durability sufficient to survive under severe conditions. Living entities such as yeasts, once applied should be more likely to establish permanence on leaves of potato plants than are synthetic chemicals. For this advantage to occur, yeasts should remain viable when occupying leaf surfaces. The Mp wild yeast strain could be re-isolated within a week under laboratory conditions. To combat Potato Late Blight epidemics it appears beneficial for the biocontrol agent to be present in advance of *P. infestans* inoculum. The pre-treatment of Mp within a narrow range of concentrations successfully suppressed Late Blight symptoms hypothetically, by pre-initiating a nonhost-like immunity response. The inoculum of the Mp oomycete usually arrives in a water carrier. Therefore bio-control yeasts should also be able to resist the washing action of rain or irrigation.

Suppressive agents and self-inhibitors are released in culture by many fungi (Griffin 1994). Recently it was pointed out that all fungal genomes sequenced to date include genes for the enzyme, DNase (Hadwiger and Polashock 2013). These genes also code for a SignalP peptide component on the N-terminal end of the DNase protein. This feature gives the DNase a potential for transference across membranes and thus a mechanism for release from the fungal cell as well as for entry into the plant cell. The DNase upon arrival within the plant nucleus is associated with subtle DNA fragmentation that activates some plant defense genes (PR genes) by the proposed mechanism of altering chromatin structure (Isaac et al. 2009)

Results of the present study suggest the need for further research directed toward developing procedures to deliver the optimal concentrations of the yeast biological control agents in field tests if the Late Blight disease is to be effectively managed. The advantages of controlling a major disease by microbes, without the environmental and food safety hazards that may accompany the use of conventional fungicides, can be immense both in terms of safety and economy. Thus, progression towards this anticipated end-point, justifies future efforts towards the optimization of this organism-induced disease resistance.

Conclusions

Three yeast strains were featured in this report, The Mp strain exhibited detectable suppression of Fspi in the nonhost resistance/susceptible reaction pea assay. More importantly, at the optimal concentration Mp suppressed the development of late blight symptoms to near zero. The precise mechanism by which suppression was induced was not determined and its explanation may fall within those mechanisms previously proposed for other yeasts (El-Tarabily and Sivasithamparam 2006). These include nutrient competition, direct parasitism,

induced resistance,” antibody production” and “the possession principle” against the pathogen. It is now known that the DNAs of all of the fungi sequenced to date (Hadwiger and Polashock 2013) contain the sequence of a DNase 1 type enzyme coding for a protein that can exit the fungal cell. This DNase mechanism may be added to the list. The current research addressed two ways for the yeast to influence Potato Late Blight: to directly affect the *P. infestans* spore growth or activate a plant defense response (e.g., damage DNA and/ or affect other host signaling). Since there was only minimally-detectable direct inhibition of other fungi by the wild yeast cells, the suppression observed was more likely due to the activation of a defense response.

Acknowledgments We thank Lei Zhang and Pat Okubara for the review of this manuscript. PPNS #0668, Department of Plant Pathology, College of Agricultural Human and Natural Resources Sciences, Agr. Res. Center, Hatch Project No. WNPO3847. These experiments comply with the current laws of the U.S.A. where they were performed.

References

- Bourret, T.B., G.G. Grove, G.J. Vandemark, T. Kenick-Kling, and D.A. Glawe. 2013. Diversity and molecular determination of wild yeasts in a central Washington vineyard. *North American Fungi* 8: 1–32.
- Buck, J.W. 2002. In vitro antagonism of *Botrytis cinerea* by phylloplane yeasts. *Canadian Journal of Botany* 80: 885–891.
- Chan, Z., and S. Tian. 2006. Induction of H₂O₂-metabolizing enzymes and total protein synthesis by antagonistic yeast and salicylic acid in harvested sweet cherry fruit. *Postharvest Biology and Technology* 39: 314–320.
- Cruickshank, I.A.M., and D.R. Perrin. 1963. Studies on phytoalexins VI. Pisatin: the effect of some factors on its formation in *Pisum sativum* L., and the significance of pisatin in disease resistance. *Australian Journal of Biological Sciences* 16: 111–128.
- Durrant, W.E., and X. Dong. 2004. Systemic acquired resistance. *Annual Review of Phytopathology* 42: 185–209.
- El-Tarabily, K.A., and K. Sivasithamparam. 2006. Potential of yeasts as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. *Mycoscience* 47: 25–35.
- Filonow, A.B.. 1998. Role of competition for sugars by yeasts in the biocontrol of gray mold of apple. *Biocontrol Science and Technology* 8: 243–256.
- Griffin, D.H. 1994. *Fungal physiology*, 382. NY: Wiley-Liss.
- Hadwiger, L.A. 2008. Pea-*Fusarium solani* interactions: Contributions of a system toward understanding disease resistance. *Phytopathology* 98: 372–379.
- Hadwiger, L.A., and J.M. Beckman. 1980. Chitosan as a component of pea *Fusarium* interactions. *Plant Physiology* 66: 205–211.
- Hadwiger, L.A., and J. Polashock. 2013. Fungal mitochondrial DNases: effectors with the potential to activate plant defenses in nonhost resistance. *Phytopathology* 103: 81–90.
- Hahn, M.G., and P. Albersheim. 1978. Host-Pathogen Interactions: XIV. Isolation and partial characterization of an elicitor from yeast extract. *Plant Physiology* 62: 107–111.
- Hamizah, G., T.M.M. Mahmud, S.H. Ahmad, and S. Kamarkuzaman. 2013. Screening of antagonistic yeast for biological control activity against anthracnose (*Colletotrichum gloeosporioides*) in ‘Frangi’ papaya. *Acta Horticulturae* 1012: 739–744.
- Helbig, J. 2002. Ability of the antagonistic yeast *Cryptococcus albidus* to control *Botrytis cinerea* in strawberry. *Biological Control* 47: 85–99.
- Isaac, J., S.L. Hartney, K. Druffel, and L.A. Hadwiger. 2009. The non-host disease resistance response in kpeas: alterations in phosphorylation and ubiquitination of HMG A and histones, H2A/H2B. *Plant Science* 177: 439–449.
- Jiang, F., X. Zheng, and J. Chen. 2009. Microarray analysis of gene expression profile induced by the biocontrol yeast *Cryptococcus laurentii* in cherry tomato fruit. *Gene* 430: 12–16.
- Lipke, P.N., and R. Ovalle. 1998. Cell wall architecture in yeast: New structure and new challenges. *Journal of Bacteriology* 180: 3735–3740.
- Reeleder, R.D. 2003. The use of yeasts for biological control of the plant pathogen *Sclerotinia sclerotiorum*. *Biocontrol* 49: 583–594.
- Shalaby, E.S., and F.E. Mohamed. 2008. Application of *Saccharomyces cerevisiae* as a biocontrol agent against *Fusarium* infection of sugar beet plants. *Acta Biologica Szegediensis* 52: 271–275.
- Truman, W., M.H. Bennett, I. Kubigsteltig, C. Turnbull, and M. Grant. 2007. Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. *Proceedings of the National Academy of Sciences of the United States of America* 104(1075–1080): 2007. doi:10.1073/pnas.0605423104.
- Vogel, H.J. 1956. A convenient growth medium for *Neurospora*. *Microbial Genetics Bulletin* 13: 43.
- Wan, J., S.-C. Xiang, D. Neece, K.M. Pamonell, S. Clough, S. Kim, M.G. Stacey, and G. Stacey. 2008. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. *Plant Cell* 20: 471–481.