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ORIGINAL PAPER

Evaluation of economically feasible, natural plant extract-based microbiological media for producing biomass of the dry rot biocontrol strain *Pseudomonas fluorescens* P22Y05 in liquid culture

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Abstract The production of microbial biomass in liquid media often represents an indispensable step in the research and development of bacterial and fungal strains. Costs of commercially prepared nutrient media or purified media components, however, can represent a significant hurdle to conducting research in locations where obtaining these products is difficult. A less expensive option for providing components essential to microbial growth in liquid culture is the use of extracts of fresh or dried plant products obtained by using hot water extraction techniques. A total of 13 plant extract-based media were prepared from a variety of plant fruits, pods or seeds of plant species including *Allium cepa* (red onion bulb), *Phaseolus vulgaris* (green bean pods), and *Lens culinaris* (lentil seeds). In shake flask tests, cell production by potato dry rot antagonist *Pseudomonas fluorescens* P22Y05 in plant extract-based media was generally statistically indistinguishable from that in commercially produced tryptic soy broth and nutrient broth as measured by optical density and colony forming units/ml produced ($P \leq 0.05$, Fisher's protected LSD). The efficacy of biomass produced in the best plant extract-based media or commercial media was equivalent in reducing Fusarium dry rot by 50–96 % compared to controls. In studies using a high-throughput microbioreactor, logarithmic growth of P22Y05 in plant extract-based media initiated in 3–5 h in most cases but specific growth rate and the time of maximum OD varied as did the maximum pH obtained in media. Nutrient analysis of selected media before and after cell growth indicated that nitrogen in the form of NH_4 accumulated in culture supernatants, possibly due to unbalanced growth conditions brought on by a scarcity of simple sugars in the media tested. The potential of plant extract-based media to economically produce biomass of microbes active in reducing plant disease is considerable and deserves further research.

Keywords Microbioreactor · Plant extract-based media · Biological control · Dry rot of potato

Introduction

The production of microbial biomass in liquid culture often represents a mandatory step prior to or as part of conducting experimentation with isolates of bacteria or yeast (Slininger et al. 1994). Typically, the production of media for such studies consists of utilizing commercially produced powdered media (Masi et al. 2014) or media composed of specific quantities of purified carbon, nitrogen, and nutrient sources (Kobori et al. 2015). However, the costs of these commercially prepared nutrient media or purified media components are prohibitive and can represent a significant hurdle to the conduct of research in locations where obtaining these products is difficult. Alternative, less expensive sources of carbon, nitrogen and other nutrients can be considered to offset the costs of

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liquid production media and include, but are not limited to, agricultural waste stream products [such as soybean waste, corn steep liquor, molasses and distillers' solubles (Gudiña et al. 2015; Plaga et al. 1989; Wong and Lay 1980)], oil seed meals (Sankh et al. 2011), and seed extracts (Chaturvedi et al. 1991). Another less often considered option for providing components essential to microbial growth is the use of extracts of fresh plant products or byproducts obtained by using hot water extraction techniques. While plant-based materials can provide many opportunities for developing inexpensive media for specialized or general microbial growth (Charalampopoulos et al. 2002; Marone et al. 2014; Zaid et al. 2012) the literature is limited on how such alternative media would perform in producing cells of a biological control agent regarding the quantity and efficacy of the biomass produced.

The use of biological control agents to reduce plant disease has great potential in developing countries (Pereg and McMillan 2015), especially if lower cost liquid media could be developed for conducting research on producing microbial biomass of biocontrol agents. Postharvest disease of harvested commodities results in estimated losses of 5 % to more than 20 % in industrialized countries and can be as high as 50 % in developing countries (Eckert and Ogawa 1985; Janisiewicz and Korsten 2002; Nunes 2012). Due to restrictions in the use of chemicals on harvested products, biological control treatments can be especially useful against postharvest disease.

Gibberella pulicaris (Fr.:Fr.) Sacc. (anamorph:*Fusarium sambucinum* Fuckel), is a serious pathogen in potato tuber storages. We have demonstrated the utility of a number of Gram negative bacteria in reducing this disease (Schisler et al. 1997). *Pseudomonas fluorescens* strain P22Y05 [= NRRL B-21053, Agricultural Research Service Culture Collection (NRRL), NCAUR, Peoria, Illinois, USA] can be especially effective against dry rot but nothing is known regarding its amenability to growth in plant extract-based media nor the bioefficacy of cells grown in such media.

The production of cells of bacteria or yeasts in nutrient broths contained in Erlenmeyer flasks has been a research norm for decades. Recently, a new technology consisting of a microbioreactor where microbial growth takes place in microtiter plates allows growth as measured by optical density, and medium conditions to be automatically recorded without interrupting growth to take samples (Huber et al. 2009; Kensy et al. 2009). The suitability of this technology to compare microbial growth on plant extract-derived media and the biocontrol efficacy of the cells produced has not been previously documented.

The objectives of this work were to (1) prepare a range of microbiological media using extracts from a variety of fresh and dried plant part sources, (2) evaluate the quantity and biocontrol efficacy of bacterial biomass produced in the plant extract-based media compared to commercially available media, and (3) compare and characterize the growth and efficacy of the bacterial biocontrol agent when grown in plant extract-based and commercial media in shake flask versus microbioreactor cultures.

Materials and methods

Bacterial culture

A pure culture of *P. fluorescens* biovar 5 (P22Y05, NRRL B-21053) was used in this study. This strain has previously been reported for its biocontrol activity against Fusarium dry rot incited by *G. pulicaris* on potatoes in storage (Schisler and Slininger 1994). Stock cultures of isolate P22Y05 were maintained in 20 % glycerol in cryovials stored at −80 °C. Stock cultures were streaked on 1/5 strength trypticase soy broth agar plates (1/5 TSA; Difco Laboratories, Detroit, MI), checked for purity and subcultured before every experiment.

Selection and source of plant based products

Different plant based products such as vegetables, fruits, and seeds were selected on the basis of their nutritional values, cost and seasonal availability and collected from local markets in Peoria, IL. Perishable items were either used fresh or kept at −4 °C to avoid spoilage prior to use. Non-perishable products (such as lentil seeds) were stored at room temperature. During the course of these studies, a total of thirteen plant extract-based media were evaluated and included the following plant species and plant part utilized for extracts: *Solanum tuberosum* (potato tuber), *Allium cepa* (red onion bulb), *Phaseolus vulgaris* (green bean pods), *Vigna unguiculata* (black eye bean pods), *Zea mays* (fresh sweet corn kernels), *Ipomoea batatas* (sweet potato tuber), *Lens culinaris* (red and yellow lentils and whole green lentils). Additionally, several plant extract products were tested at increased concentrations after favorable results of microbial growth and efficacy in reducing Fusarium dry rot in laboratory bioassays were obtained.

Preparation of extracts

Extracts of fruits, vegetables and lentil products were prepared by decoction using a double boiler covered with aluminum foil. Extraction was carried out by heating plant products and water contained in 1L Pyrex glass bottles to 95 °C for 45 min for vegetable and fruit products while lentils were kept at the same temperature for 55 min. Time and temperatures utilized were based on preliminary results

of colony forming units (CFU) per ml production by *Pseudomonas* spp. in media prepared using a variety of extraction times and temperatures (unpublished results). Extracts other than lentils were filtered through Whatman #1 filters (Sigma-Aldrich Corp., St. Louis, MO, USA) while lentils were filtered instead through muslin cloth due to filters rapidly clogging with plant matter. All the extracts were autoclaved for 20 min and stored at −4 °C until use. Vegetables such as onions, bean and potato were cut into 1 cm cubic pieces and water added in various ratios to optimize the concentration. In most tests, 10 or 20 g substrate per 100 ml of water were used for decoction and growth experiments. In later tests, demonstrated success in biomass production in media composed of clear extracts such as bean and onion justified testing 40 g of these plants per 100 ml of water, while lentils extracts lost clarity at concentrations higher than 20 g per 100 ml. The pH of the plant extract-based media was not adjusted. Commercially produced media of nutrient broth (NB) and tryptic soy broth (TSB) (Becton, Dickson and Company, Sparks, MD) were prepared at label rate (NB) or 20 and 100 % of label rate (TSB).

Shake-flask cultivations of strain P22Y05

For precultivation inoculum preparation of P22Y05, 25 ml of each plant extract-based and control media (NB and TSB) in 100 ml Erlenmeyer flasks were inoculated with cells grown for 24 h at 25 °C on Petri plates of 1/5 TSA. Precultures were removed from shaker incubators after 24 h at 250 rpm (2.5 cm eccentricity) and 25 °C and used to inoculate test cultures of 50 ml of medium in 250 ml flask to an initial OD_{600} of 0.1. The flasks were shaken at 25 °C and 250 rpm. After 48 h, growth was assessed by measuring optical density at 600 nm (OD_{600}) and CFU per ml by plating serial dilutions of each colonized liquid medium onto 1/5 TSA. All cultivation experiments were conducted at least twice with three replicate test culture flasks per treatment and results averaged across experiments.

Wounded potato bioassay of P22Y05 efficacy

After production in plant extract-based or commercially produced media, flasks containing cells of P22Y05 were placed on ice for 2–3 h and then tested for efficacy against *G. pulicaris* strain R-6380 using a wounded potato bioassay (Schisler and Slininger 1994). Cells of P22Y05 from test cultures were combined 1:1 (v/v) with *G. pulicaris* R-6380 at 5×10^5 conidia per ml (by hemacytometer count). Five µl of this mixed inoculum was then added to potato wounds made with a 2 mm diameter × 2 mm length steel pin. Each treatment was repeated on eight to ten size B (approximately 5 cm diameter) Russet Norkotah, Red Norland and Russet Burbank washed seed potatoes (Wisconsin Seed Potato Certification Program, University of Wisconsin Madison, Antigo, WI) kept until 1 day before assays in a cold room at ~4 °C. Each potato had four wounds around its circumference, with three wounds receiving different P22Y05 and pathogen mixed inoculum treatments and one control wound receiving only pathogen mixed with weak buffer (pH 7.2, 0.004 % [wt/v] KH_2PO_4 buffer with 0.019 % [wt/v] $MgCl_2$). Each potato then was placed in a plastic weigh boat containing a dry 2.54 cm-cut square of WypAll L40 all-purpose wiper paper towel (Kimberly-Clark Worldwide, Inc., Neenah, WI). Boats were moved to trays that were supplied with two dry WypAlls over the top of potatoes and two Wypalls wet with 40 ml of water each and placed on either side of the tray, plastic bagged, and stored 21 days at 15 °C. Dry rot then was evaluated by quartering each potato by slicing through the center of each of the four wounds. The extent of disease in each wound was rated by adding the greatest depth and width measurements (mm) of discolored necrotic tissue extending below and to the sides of the wound. Relative disease (%) was calculated as 100 × (wound disease rating/average disease rating of wounds receiving pathogen only).

Assessment of plant extract-based media in shake flasks

An initial selection of six plant extract-based media, from four different biomass sources, and TSB 20 % was grown in flasks as described above (Table 1 lists the plant extract media assayed) to determine OD_{600}, CFU per ml and overall efficacy of strain P22Y05 against Fusarium dry rot when assayed on Russet Norkotah, Red Norland and Russet Burbank tubers. Efficacy results were pooled across all potato cultivars. Due to satisfactory results with these six initial extracts, they were included in a second series of tests that also included six new extracts and TSB assayed at 20 and 100 % for a total of 14 treatments from seven different biomass sources (see Table 2 for a list of retained and new treatments). The efficacy of P22Y05 biomass produced in each test medium was evaluated against each of the three potato cultivars separately and then an overall performance across all cultivars was calculated.

Comparison of selected plant extract-based media in flasks versus microbioreator plates

Promising plant extract media from earlier tests, as well as nutrient broth and tryptic soy broth at 100 % label rates were utilized to grow cells of P22Y05 in shake flasks and in BioLector® microbioreactor system (m2p-labs, Aachen,

Table 1 Cell viability and efficacy of antagonist *Pseudomonas fluorescens* P22Y05 in reducing Fusarium dry rot when grown in an initial selection of plant-extract-based media

Plant extract/medium	Preparation (%)	Strain performance		
		OD (600 nm)	CFU/ml (average)	Efficacy (% change vs. control)
Corn	20	0.54[E]	2.25×10^{9} [E]	−12.9[AB]
Onion	10	0.74[C]	4.75×10^{9} [BCD]	−18.7[AB]
Onion	20	1.56[A]	7.42×10^{9} [A]	−74.8[B]
Bean	20	0.82[B]	6.67×10^{9} [AB]	−62.5[AB]
Potato	10	0.44[F]	4.00×10^{9} [D]	−71.4[B]
Potato	20	0.63[D]	3.25×10^{9} [D]	−68.9[B]
TSB	20	0.64[D]	4.79×10^{9} [CD]	−61.4[AB]
F. sam. only	–	–	–	0.0[A]

[A] Cell viability evaluated after 48 h growth at 250 rpm in 50 ml of liquid medium in 250 ml non-baffled Erlenmeyer flasks

[B] Efficacy results were pooled over three different potato cultivars and represent the change in dry rot lesion size compared to the positive control

[C] Within a column, means not followed by the same letter are significantly different ($P \leq 0.05$, Fisher's Protected LSD test, FPLSD). Mean separation of CFU/ml data is based on analysis of $\log_{10}$-transformed values if transformation improved data normality. Non-transformed values are presented

[D] *OD* optical density; *CFU* colony forming units

[E] *Preparation* percentage (weight/volume) of plant biomass extracted in hot water prior to filtering and use as a microbial growth medium

[F] *TSB 20 %* Tryptic soy broth prepared at 20 % of the label rate

[G] *F. sam. Fusarium sambucinum* control without treatment with antagonist strain P22Y05

Germany) 48 well, baffled flower plates (MTP-48-BOH) (see Table 3 for a treatment list). In preparation of initiating cell growth under both cultivation methods, precultures of strain P22Y05 were grown as described earlier. Three replicate test cultures for each test medium were prepared and incubated in a shaker incubator as described above. Additional test cultures were prepared for each medium and the inoculated broths immediately used to fill a Biolector microbioreactor 48 well flower plate (MTP-48-BOH). Inoculated medium from a test culture flask was transferred aseptically to Flower plate wells using a sterile pipette. Four to six replicate wells filled with 500 µl per well were prepared per treatment. Plates then were covered with a gas permeable sealing foil with an evaporation reducing layer (m2p-labs, Baesweiler, Germany). Cultivation conditions for the Biolector were 25 °C, 1100 rpm with a circular eccentricity of 3 mm diameter, and relative humidity of approximately 90–95 %. The Biomass concentration was monitored via back-scattered light at a wavelength of 600 nm. Final readings for each medium type over the course of an experiment were obtained by subtracting back-scattered light readings obtained from each uninoculated medium. The light scatter readings were measured every 30 min using a gain factor setting of 10 for the signal amplification of the photomultiplier. Light scatter readings were converted to OD_{600} values via the preparation of a calibration curve for light scatter and OD_{600} readings taken for a series of concentrations of cells of strain P22Y05. After harvest at 48 h, cells from flower plates and shake flasks were harvested and used to determine CFU per ml and bioefficacy against Fusarium dry rot on potato tubers as described earlier.

Changes in pH and OD_{600} over time during growth of P22Y05 in microbioreactor

Six plant extract-based media from the shake flask versus microbioreactor study, along with TSB 100 % and NB 100 % were selected for evaluation of pH and OD_{600} in bioreactor plates containing optode sensors for pH in each well. Preparation of test cultures of each medium and flower plate inoculation was as described earlier. Readings for pH and backscatter light were recorded every 15 and 20 min, respectively, over 48 h with pH and backscatter gain factor settings of 30 and 10 utilized, respectively. Specific growth rate (h^{-1}) of P22Y05 in each medium was calculated from data obtained during early exponential growth.

Data analysis and presentation

All experiments were conducted at least twice. Growth and efficacy data were combined and analyzed using one-way analysis of variance (ANOVA). For CFU per ml data, $\log_{10}$-transformed values were subject to ANOVA and mean separation if the transformation improved data

Table 2 Cell viability and efficacy of antagonist *Pseudomonas fluorescens* P22Y05 in reducing Fusarium dry rot on three potato cultivars when grown in a second set of plant-extract-based media

Plant extract/ medium	Preparation (%)	Strain performance					
		OD (600 nm)	CFU/ml (average)	Efficacy (% change vs. control)			
				Potato cultivar			
				Russet Norkotah	Red Norland	Russet Burbank	All cultivars (average)
Corn	20	0.40^{J}	$1.67 \times 10^{9\ F}$	-78.4^{B}	-86.7^{BC}	-96.1^{B}	-87.0^{C}
Onion	10	0.73^{FG}	$5.17 \times 10^{9\ DE}$	-78.4^{B}	-97.4^{C}	-91.9^{B}	-89.2^{C}
TSB	20	0.61^{HI}	$4.00 \times 10^{9\ E}$	-85.4^{B}	-95.8^{BC}	-89.0^{B}	-90.0^{C}
Bean	20	0.82^{F}	$5.50 \times 10^{9\ CDE}$	-71.4^{B}	-88.9^{BC}	-95.6^{B}	-85.3^{BC}
Potato	20	0.67^{GH}	$4.00 \times 10^{9\ E}$	-90.2^{B}	-82.8^{BC}	-95.8^{B}	-89.6^{C}
Potato	10	0.44^{J}	$4.25 \times 10^{9\ E}$	-63.7^{B}	-74.9^{BC}	-93.5^{B}	-77.4^{BC}
Onion	20	1.49^{D}	$7.17 \times 10^{9\ BC}$	-78.4^{B}	-84.4^{BC}	-90.6^{B}	-84.5^{BC}
Sweet potato	20	0.56^{I}	$4.67 \times 10^{9\ E}$	-81.8^{B}	-87.0^{BC}	-92.4^{B}	-87.0^{C}
TSB	100	4.79^{A}	$1.16 \times 10^{10\ A}$	-71.4^{B}	-92.5^{BC}	-94.8^{B}	-86.0^{C}
Black eye bean	20	1.19^{E}	$4.58 \times 10^{9\ E}$	-57.0^{B}	-68.8^{B}	-75.7^{B}	-67.2^{B}
Whole lentils	20	2.75^{C}	$6.58 \times 10^{9\ CD}$	-81.0^{B}	-77.4^{BC}	-90.4^{B}	-82.9^{BC}
Yellow lentils	20	0.45^{J}	$4.58 \times 10^{8\ F}$	-81.5^{B}	-86.7^{BC}	-89.8^{B}	-86.0^{BC}
Red lentils	20	3.09^{B}	$6.92 \times 10^{9\ C}$	-77.5^{B}	-84.2^{BC}	-91.2^{B}	-84.3^{BC}
Onion	40	2.83^{C}	$8.83 \times 10^{9\ B}$	-80.0^{B}	-87.0^{BC}	-89.8^{B}	-85.6^{BC}
F. sam. only	–	–	–	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}

[A] Cell viability evaluated after 48 h growth at 250 rpm in 50 ml of liquid medium in 250 ml non-baffled Erlenmeyer flasks

[B] Efficacy results were pooled over two trials using three different potato cultivars and represent the change in dry rot lesion size compared to the positive control

[C] Within a column, means not followed by the same letter are significantly different ($P \leq 0.05$, Fisher's Protected LSD test, FPLSD). Mean separation of CFU/ml data is based on analysis of $\log_{10}$ transformed values if transformation improved data normality. Non-transformed values are presented

[D] *OD* Optical density; *CFU* Colony forming units

[E] *Preparation* percentage (wt./vol.) of plant biomass extracted in hot water prior to filtering and use as a microbial growth medium

[F] *TSB* Tryptic soy broth prepared at 20 or 100 % of the label rate

[G] *F. sam. Fusarium sambucinum* control without treatment with antagonist strain P22Y05

normality. Non-transformed values are reported. Prior to pooling efficacy data across cultivars, cultivar by treatment interactions were calculated. Separation of means was accomplished using Fisher's protected LSD test ($P \leq 0.05$) (Statistix 10, Tallahassee, FL). Pearson Product Moment and Spearman Rank Order tests were run to determine if CFU and optical density measurements for treatments were correlated. Experiments to determine pH and OD_{600} of inoculated media over time were conducted twice and representative pH and growth curves plotted (Figs. 1, 2).

Chemical analysis of media

The chemical analysis of plant extract-based and commercial media before and after 48 h cultivation of strain P22Y05 in shake flasks was conducted by the Cornell Nutrient Analysis laboratory, Cornell University, Ithaca, New York except for the determination of simple sugars. Cells were separated from 48 h cultures by centrifugation (4070*g*, Sorvall Instruments RC5C centrifuge, Dupont, Wilmington, DE) prior to chemical analysis. Simple sugar content in the liquid media was determined using a microplate modification of the phenol/sulfuric acid-based colorimetric method (Chow and Landhäusser 2004; Masuko et al. 2005). A calibration curve for the assay was prepared using a mixture of glucose, fructose and galactose in a ratio of (1:1:1). Samples of various concentrations of the sugar mixture and fresh or spent plant extract-based and commercial media were analyzed. Fifty microliters of test liquid was added to wells of 96 well

Table 3 Comparison of cell viability and efficacy of antagonist *Pseudomonas fluorescens* P22Y05 in reducing Fusarium dry rot when grown in a third selection of plant extract-based media in Erlenmeyer flasks or Biolector® microbioreactor system plates

Plant extract/medium	Preparation (%)	Strain performance			
		CFU/ml (average)		Efficacy (% change vs. control)	
		Flask	Bioreactor	Flask	Bioreactor
Onion	20	7.63×10^{8C}	1.29×10^{10A}*	-79.1^{C}	-79.8^{B}
Onion	40	1.01×10^{9C}	2.29×10^{9B}*	-76.7^{C}	-85.6^{B}*
Bean	20	6.80×10^{8C}	3.86×10^{9B}	-81.8^{C}	-80.5^{B}
Bean	40	8.33×10^{8C}	2.15×10^{9BC}*	-51.2^{B}	-70.7^{B}
Whole green lentils	20	1.23×10^{10A}	1.20×10^{9BC}*	-81.8^{C}	-80.7^{B}
Red lentils	20	1.05×10^{9C}	7.00×10^{7D}*	-71.9^{BC}	-85.7^{B}*
TSB	100	6.04×10^{9B}	1.01×10^{9C}	-72.1^{BC}	-87.2^{B}*
NB	100	2.85×10^{8D}	4.90×10^{7D}*	-73.1^{BC}	-82.2^{B}
F. sam. only		–	–	0.0^{A}	0.0^{A}

[A] Cell viability as measured by colony forming units per ml (CFU per ml) was evaluated after 48 h growth in 50 ml of liquid medium in 250 ml non-baffled Erlenmeyer flasks or in Biolector® microbioreactor system plates

[B] Efficacy results were pooled over two trials using three different potato cultivars and represent the change in dry rot lesion size compared to the positive control

[C] Within a column, means not followed by the same letter are significantly different ($P \leq 0.05$, Fisher's Protected LSD test, FPLSD)

[D] Within a row, an asterisk by a CFU per ml or efficacy value indicates a significant difference between flask and bioreactor obtained means ($P \leq 0.05$, FPLSD)

[E] Mean separation of CFU per ml data is based on analysis of $\log_{10}$ transformed values if transformation improved data normality. Non-transformed values are presented

[F] *CFU* Colony forming units

[G] *Preparation* percentage (wt./vol.) of plant biomass extracted in hot water prior to filtering and use as a microbial growth medium

[H] *TSB* tryptic soy broth prepared at 100 % of the label rate; *NB* Nutrient broth prepared at 100 % of the label rate

[I] *F. sam. Fusarium sambucinum* control without treatment with antagonist strain P22Y05

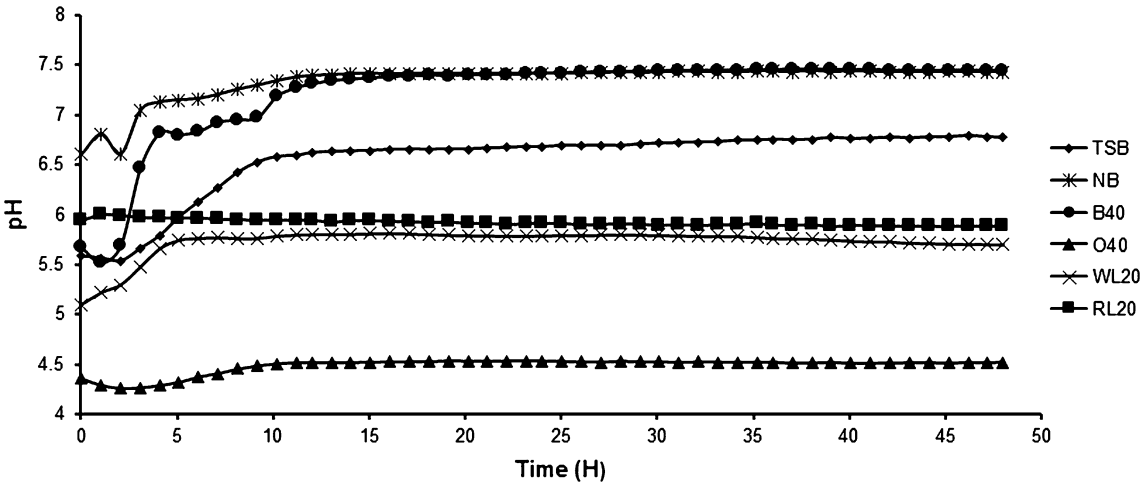

Fig. 1 pH change in commercially produced and top performing plant extract-based liquid media during 48 h of growth of antagonist *Pseduomonas fluorescens* P22Y05. TSB, tryptic soy broth prepared at label rate; NB, nutrient broth prepared at label rate; B40, bean extract 40 % medium; O40, onion extract 40 % medium; WL20, whole green lentil extract 20 % medium; RL20, red lentil extract 20 % medium

microtiter plates followed by the addition of 150 µl of 96 % H_2SO_4. After plates were shaken for 5 min, 50 µl of 5 % phenol in water was added to the wells and the mixture heated for 5 min at 90 C in a water bath. Plates then were cooled at room temperature, their surfaces dried and OD_{490nm} determined using a microplate reader (Power Wave XS, Microplate spectrophotometer, BioTek, Winooski, VT, USA).

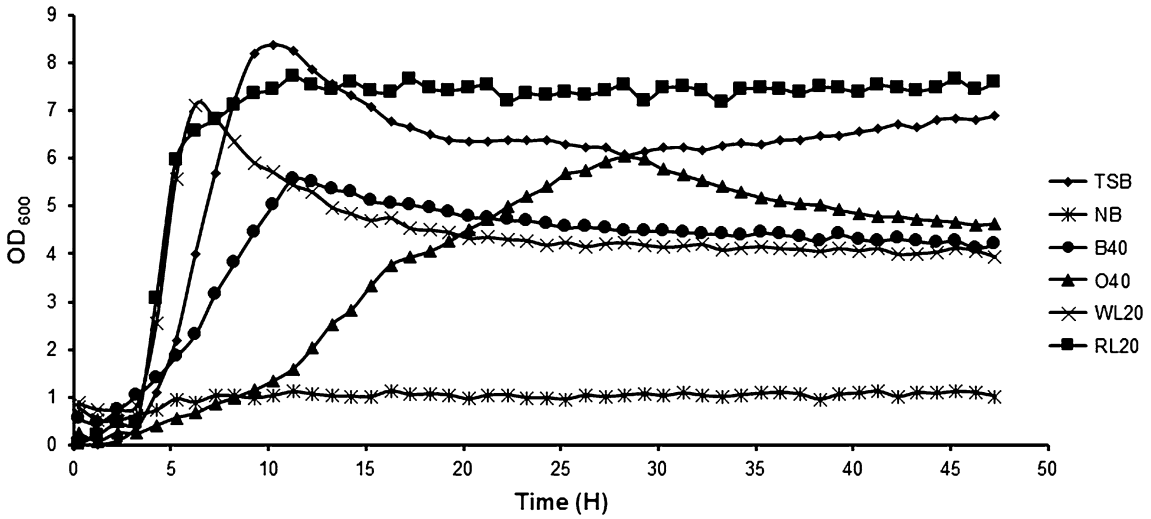

Fig. 2 Optical density change (OD_{620}) over 48 h of growth of antagonist *Pseduomonas fluorescens* P22Y05 in commercially produced and top performing plant extract-based liquid media. Final readings for each medium type over the course of an experiment were obtained by subtracting OD_{620} readings obtained from each uninoculated medium. TSB, tryptic soy broth prepared at label rate; NB, nutrient broth prepared at label rate; B40, bean extract 40 % medium; O40, onion extract 40 % medium; WL20, whole green lentil extract 20 % medium; RL20, red lentil extract 20 % medium

Results

Performance of strain P22Y05 in plant extract-based liquid media

After growth of strain P22Y05 in shake flasks for 48 h, OD_{600} values obtained in the media tested differed significantly with onion 20 % and bean 20 % media supporting the highest values (1.56 and 0.82, respectively) and corn 20 % and potato 10 % the lowest (0.54 and 0.44, respectively; $P \leq 0.05$, Fisher's protected LSD; Table 1). Trends in media performance were similar for CFU produced per ml. The onion 20 % and bean 20 % media produced the greatest number of viable cells (7.42×10^9 and 6.67×10^9, respectively) and significantly more than produced in the corn 20 % and potato 10 % media (Table 1). The CFU and optical density measurements for treatments were found to be significantly correlated using both the Pearson Product Moment ($P = 0.03$) and Spearman Rank Order ($P = 0.006$) tests. Cell production in the best plant extract-based media was higher than produced in the commercially produced Tryptic Soy Broth 20 % medium (Table 1). Cells from the two potato extract media and onion at 20 % reduced Fusarium dry rot compared to the pathogen only in results pooled across three potato cultivars ($P \leq 0.05$, Fisher's protected LSD; Table 1). Cells produced in all plant extract-based media did not differ from cells produced in TSB 20 % medium in reducing dry rot (Table 1). It is interesting to note that there was not a significant correlation between the efficacy and the number of cells produced, either based on optical density or CFU ($P > 0.48$ and >0.26, respectively).

Performance of strain P22Y05 in a second set of plant extract-based liquid media

Plant extract based media from the first selection experiments and newly assayed extracts varied significantly ($P \leq 0.05$) in both the OD_{600} and CFU per ml values obtained for P22Y05 growth in shake flasks for 48 h (Table 2). The highest OD_{600} values were for red lentils 20 %, onion 40 % and whole green lentils 20 % (3.09, 2.83, and 2.75, respectively) while the lowest were for corn 20 %, yellow lentils 20 % and sweet potato 20 % (0.40, 0.45, 0.56, respectively). The commercially produced TSB 20 % medium produced one of the lowest OD_{600} values (0.61) after growth of P22Y05 while the TSB 100 % medium produced the highest (4.79; $P \leq 0.05$, Table 2). Similarly, extract-based media vary significantly in the CFU per ml produced with the corn 20 % and yellow lentil 20 % being the lowest (1.67×10^9 and 4.58×10^8, respectively) and onion 40 %, onion 20 % and red lentils 20 % being the highest (8.83×10^9, 7.17×10^9 and 6.92×10^9, respectively). The TSB based media were superior (1.16×10^{10}) or among the lowest values obtained depending on whether tested at 100 or 20 % levels, respectively (Table 2). As in the earlier experiment, the CFU and optical density measurements for treatments were found to be significantly correlated ($P < 0.001$) using both the Pearson Product Moment and Spearman Rank Order tests.

Cells of P22Y05 grown in the plant extract-based media or in the commercially produced TSB media reduced Fusarium dry rot by 57–90, 69–97 and 76–96 % compared to the control for bioassays conducted on Russet Norkotah, Red Norland, and Russet Burbank tubers, respectively

(Table 2). Interactions between cultivars and treatments were not significant. When results were pooled across all cultivars, cells grown in all plant extract-based media performed equivalently in reducing Fusarium dry rot compared to both of the TSB-based media, with the exception of cells grown in black-eye bean media not being as efficacious. As in the first flask experiment, there was not a significant correlation between the efficacy and number of cells produced, either based on optical density or CFU ($P > 0.22$ and > 0.20, respectively).

Several plant extract-based media chosen from the second selection experiments as well as TSB 100 % and nutrient broth 100 % (NB) media varied significantly when P22Y05 cells were grown in shake flasks or in microbioreator plates. In shake flask cell production, whole green lentils 20 % medium produced a greater CFU per ml than TSB 100 % ($P \leq 0.05$, Table 3) and both produced higher CFU per ml than any other plant extract-based medium. Production in NB 100 % was lower than any other treatment. In the microbioreactor plates, onion 20 %, bean 20 % and onion 40 % produced the greatest CFU per ml and more CFU per ml than the TSB 100 % medium ($P \leq 0.05$, Table 3). Red lentils 20 % and NB 100 % produced less CFU per ml than the other media.

Cells of strain P22Y05 produced in all the media tested reduced Fusarium dry rot in pooled results across three potato cultivars compared to the pooled control regardless of whether produced in shake flasks (reductions of 51.2–81.8 %) or microbioreactors (reductions of 70.7–87.2 %) ($P \leq 0.05$, Table 3). Cells produced in any of the plant extract-based media under either production method did not differ in efficacy from cells produced in TSB 100 % or NB 100 % (Table 3). For TSB 100 %, red lentils 20 % and onion 40 % media, microbioreactor-produced cells were more effective in reducing dry rot than cells produced in shake flasks (Table 3). Pearson Product Moment and Spearman Rank Order analyses indicated that the following quantities were not significantly correlated (i.e., not predictive of one another): flask and bioreactor cell productions (CFU per ml) ($P > 0.57$), flask cell production and disease control ($P > 0.47$), bioreactor cell production and disease control ($P > 0.1$), and disease control observed in flask produced cells and disease control observed in bioreactor produced cells ($P > 0.117$).

Changes in pH and OD_{600} over time during growth of P22Y05 in microbioreactor

Average pH values of media as measured in bioreactor plates varied both in initial pH and in pH over the course of the 48 h of growth of P22Y05 (Fig. 1). Five hours after inoculation, the pH of NB 100 %, bean 40 %, TSB 100 % and whole green lentils 20 % had risen from initial values (Fig. 1). By 15 h, all extract pH values had stabilized with the highest pH of $\sim$7.4 in bean 40 % and whole green lentil 20 % media and the lowest pH of $\sim$4.5 in onion 40 % medium.

The scattered light values (converted to OD_{600} in Fig. 2) for these same media indicated logarithmic growth initiated about 3–5 h after inoculation for all the media except NB 100 % which showed minimal increase in scattered light values (Fig. 2). Specific growth (h^{-1}) values for TSB, NB, bean 40 %, onion 40 %, whole green lentil 20 % and red lentil 20 % were 0.57, 0.09, 0.13, 0.19, 0.42 and 0.46, respectively. The maximum scattered light readings occurred in TSB 100 % and red lentil 20 % media at 10–11 h ($\sim$8.4 and $\sim$7.8, respectively). Cells in whole green lentil 20 % reached a maximum reading after 6 h, faster than any other medium while onion 40 % medium reached an OD_{600} maximum of $\sim$6.0 after 28 h, slower than any other medium by 15 h (Fig. 2). Moderate decreases in scattered light readings were commonly observed after maximum values were obtained.

Chemical analysis of media

Readings of Ca, Fe, Mg, P were higher in medium before biomass production than after in almost every case (Table 4). Fe content was the highest in red lentil medium before production of P22Y05 cells. Red lentil medium was also the highest of the extract-based media in Cu, Mo and Zn content. In every case, levels of nitrogen as NH_4–N increased in media after the cultivation of P22Y05 (Table 4). Red lentil 20 % and TSB 100 % had the highest levels of NH_4–N before cultivation (52 and 53 mg/l, respectively) and after (662 and 644 mg/l, respectively).

Discussion

Multiple sources of plant extracts used to produce microbiological media often performed as well as two commonly used, commercially produced powdered media (nutrient broth and tryptic soy broth) as measured by the growth and efficacy (Tables 1, 2, 3) of *P. fluorescens* P22Y05, a biocontrol strain effective against Fusarium dry rot on stored potato tubers. In experiments where the best plant extract-based media identified were used to produce P22Y05 in shake flasks and microbioreactors, cell CFU per ml production in whole green lentils 20 % medium was higher than in TSB and NB 100 % in shake flasks and higher in onion 20 %, bean 20 %, and onion 40 % media in microbioreactors (Table 3). Our results agree in concept with Agil et al. (2013) who demonstrated relatively small amounts of plant-derived amendments to liquid growth media can improve microbial fermentations.

Table 4 Chemical analysis of selected plant extract-based and commercial media after 48 h growth of *Psedomonas fluorescens* P22Y05 and when uncultured

Plant extract/media	Nutrient Analysis														
	Ca	Fe	K	Mg	P	S	Na	Co	Cu	Mn	Mo	Zn	NH_4–N	NO_3–N	Sugars
TSB 100 % (C)	3.8	0.1	1347	4.5	586.9	150.5	8037.3	0.02	0.03	0.01	0.01	0.42	644.1	0.00	0.6
TSB 100 % (UC)	5.4	0.5	1421	10.4	730.1	157.5	8387.9	0.02	0.03	0.02	0.02	0.54	53.3	0.00	1.3
NB 100 % (C)	0.5	0.1	1563	0.9	54.3	34.3	535.9	0.01	0.07	0.00	0.00	0.01	198.1	0.33	0.07
NB 100 % (UC)	1.0	0.2	274	2.3	65.4	35.3	514.0	0.01	0.04	0.00	0.00	0.02	11.5	0.00	0.10
Bean 40 % (C)	16.9	0.2	620	26.8	26.0	24.8	5.9	0.01	0.09	0.20	0.01	0.44	46.5	0.00	4.8
Bean 40 % (UC)	11.8	0.7	612	31.1	64.4	25.6	8.0	0.01	0.10	0.27	0.01	0.50	22.0	1.47	11.9
ON 40 % (C)	31.3	0.1	283	15.9	39.0	133.4	47.6	0.01	0.05	0.13	0.00	0.25	120.8	0.00	16.8
ON 40 % (UC)	31.1	0.2	343	16.0	57.2	139.2	48.6	0.01	0.04	0.13	0.00	0.25	30.3	0.00	21.2
WL 20 % (C)	2.1	0.6	1552	31.6	166.5	195.4	15.0	0.02	0.50	0.05	0.02	1.66	430.0	0.00	5.6
WL 20 % (UC)	16.2	0.8	1405	83.4	269.7	164.6	13.3	0.02	0.38	0.38	0.01	1.65	38.7	0.00	12.1
RL 20 % (C)	1.5	0.5	341	38.1	405.0	189.2	23.7	0.02	0.57	0.09	0.49	2.72	662.4	0.00	10.5
RL 20 % (UC)	8.0	2.8	1385	75.4	440.7	149.6	18.9	0.02	0.46	0.53	0.51	2.07	52.1	0.00	13.4

[A] Commercial media and plant extract-based media abbreviations: *TSB 100 %* tryptic soy broth prepared per manufacturer's instructions; *NB 100 %* nutrient broth prepared per manufacturer's instructions; *Bean 40 %* 40 % bean biomass hot water-extracted medium; *ON 40 %* 40 % onion biomass hot water-extracted medium; *WL 20 %* 20 % whole green lentil biomass hot water-extracted medium; *RL 40 %* 40 % red lentil biomass hot water-extracted medium

[B] *(C)* Cultivated medium supernate after cultivation of cells of P22Y05; *(UC)* uncultivated medium

[C] All amounts are in mg/L except "sugars" which are in g/L

[D] *NH_4–N* Nitrogen in ammonium form; *NO_3–N* nitrogen in nitrate form

[E] *Sugars* estimated amounts of available simple sugars using a phenol/sulfuric acid-based colorimetric analysis method

The efficacy of cells of P22Y05 produced in plant extract-based media in reducing Fusarium dry rot was consistently statistically equivalent to cells produced in commercial media (Tables 1, 2, 3). Related to this, we note that the level of cell production, and consequently biocontrol agent inoculum strength, varied among plant extracts and was not predictive of the level of disease suppression measured. This finding suggests that other factors had greater influence than the number of cells of strain P22Y05 on the level of disease control obtained. Such factors could include, for example, the production of antifungal compounds (Burkhead et al. 1995), the presence/production of cell protective compounds facilitating cell desiccation survival, or the induction of enzyme systems in the cells that would allow them to function optimally once delivered to the potato surface environment. While the precise mechanism of how various plant extracts may have influenced biocontrol results, variations in their nutritional composition (minerals, C and N levels and sources) have been observed (Table 4) and could be contributing to changes relating to all three factors proposed above, which in turn could impact biocontrol. Also growth and conditioning of biocontrol agents on plant extracts similar to where biocontrol agents will be deployed may boost needed enzyme systems supporting competitiveness and disease suppression. In this study, even though potato extracts did not always yield the highest cell numbers, the cells produced were among the most efficacious in reducing disease.

Our results demonstrate that plant extract-based media can support the production of an amount and quality of cells of biocontrol strain P22Y05 that is equivalent to that produced in commercially available liquid culture growth media. Microbial growth characteristics in liquid culture can vary considerably depending on the genus, species and even isolate of a species being cultured (Adiyaman et al. 2011; Slininger et al. 1994). Given the range of genera of yeasts and bacteria with reported biocontrol activity against plant pathogens, additional studies of the potentially broader utility of plant extract-based media for producing a range of biocontrol isolates is warranted.

Though the efficacy of cells produced in plant extract-based media tended to be consistently high among sets of experiments, several media varied from one set of experiments to another in the CFU per ml produced in flasks (Table 1, 2, 3) and production across all media trended lower in experiments presented in Table 3 compared to Table 2. It is likely that some variation in extracts from batch to batch contributed to this result. The feasibility of processing large volume batches of extract materials with a uniform maturity to minimize batch-to-batch variation and

assessing the consistency of plant extracts stored over time is the subject of a future study.

Strain performance regarding maximum CFU per ml obtained at 48 h of cell growth in the best plant extracts and the commercial media was not similar in shake flasks and in Biolector bioreactor tests (Table 3). Depending on the medium tested, maximum CFU per ml of strain P22Y05 was sometimes obtained in shake flasks and in other cases in the microbioreactor. Colony forming units per ml in the microbioreactor at 48 h may in some cases have dropped from maximal values if unbalanced growth conditions were reached early in the cultivation due to improved mixing and oxygen transfer in the flower plates compared to shake flasks. pH curves (Fig. 1) and growth curves (Fig. 2) generated from microbioreactor data indicated that media differed in the time of initiation and duration of logarithmic growth of P22Y05, specific growth rate, time of obtaining maximum optical density and the initial pH and magnitude of pH change detected during cultivation of P22Y05. The Biolector microbioreactor system has gained acceptance for conducting small scale studies of microbial growth kinetics (Kensy et al. 2009). While results obtained in microbioreactor plates will not always be predictive of microbial growth in large bioreactors charged with the same growth medium (Kunze et al. 2014), the utility of non-disruptively comparing growth kinetics of a single organism across multiple experimental growth media is apparent from our study.

In several of the plant extract-based media as well as the TSA broth, levels of the ammonium form of nitrogen (NH_4–N) levels were elevated after growth of strain P22Y05 compared to levels found in the broths prior to inoculation (Table 4). Consistent with this result, the pH of the majority of media was higher after growth of P22Y05 than it was prior to inoculation (Fig. 1). The highest levels of NH_4–N were in the TSB 100 % and red lentil 20 % media (Table 4). Lentils have high levels of proteins and carbohydrates primarily in the form of starch (Sánchez-Chino et al. 2015) while TSB is in large part a protein digest product. Due to the low levels of free carbohydrates in either of these media, it is reasonable to expect that available carbon in amino acids was metabolized primarily for the generation of ATP with NH_4–N from the amino acids remaining unused in media supernatants due to a lack of adequate available carbon to achieve balanced growth. Though Table 4 indicates some sugars are available in media after growth of P22Y05, carbohydrate levels estimated for the plant extract-based media, both post-cultivated and pre-cultivated, are likely to be higher than actually present. Acid treatment of structural carbohydrates and starches found in hot water extracts of plant biomass can release sugars that would erroneously register as available carbohydrates using the phenol phenol/sulfuric acid-based colorimetric method (Chow and Landhäusser 2004). The addition of inexpensive sources of sugars such as molasses or sugar cane hydrolysates (Diniz et al. 2004; Sankh et al. 2011) would likely improve nitrogen use efficiency and biomass accumulation (Srivastava et al. 2010).

Possible research approaches to further improve the growth and efficacy of strain P22Y05 in plant extract-based media are numerous and include management of medium pH (Spadaro et al. 2010; Wu et al. 2010), temperature (Pardo et al. 2005), dissolved oxygen (Tang et al. 2015) and water activity (Balamurugan et al. 2015) during cell growth. The influence of plant extract-based media on the ability of Gram negative bacteria to survive drying (Slininger et al. 2010) and rehydration processes necessary for producing dehydrated biocontrol products will need to be determined.

Our results demonstrate that several media based on extracts from onion, beans, and lentils can produce quantities of efficacious biomass of biocontrol strain *P. fluorescens* P22Y05 similar to that produced in commercially available media. The potential of plant extract-based media to produce biomass of additional microbes active in reducing plant disease is considerable and deserving of further research.

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