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Study of naturally sourced bacteria with antifungal activities

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Abstract

The study was carried out with the aim of sourcing for bacteria from the natural environment having antifungal capabilities to control and inhibit postharvest fungal spoilage of fruits and vegetables caused by *Botrytis cinerea*. Soil and water samples were collected from Heriot Watt University environment and Dr Ruth Fowler's garden and inoculated using the spread plate technique; identification was carried out using Microbact Identification kits; and isolates assayed for antifungal activities against *Botrytis cinerea*. Forty eight bacteria species were isolated out of which sixteen (16) belonging to genera Pseudomonas, Bacillus, Escherichia, Burkholderia, Staphylococcus, Streptococcus, and Proteus showed antifungal activities. Bacteria species *Pseudomonas stutzeri* and *Burkholderia cepacia* had the highest zones of inhibition with average radii of 3.06 and 3.20 cm respectively. The bacteria had the potential to inhibit mycelial and spore growth at varying levels thus making them possible candidates for further tests and studies. Considering the aim of the study, further research into identifying these antifungal isolates inhibitory compounds and metabolites is highly recommended.

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Introduction

Fungi are absolutely essential to life on the planet; their interaction with human, animal and plants has been of immense benefit in the decomposition of complex organic matter, recycling of nutrients, use as food and in food manufacture (Blackwell, 2011; Taylor *et al.*, 2004), and source of antibiotics (Buckley, 2008). Despite these benefits, plants and animals species are infected by diverse pathogenic fungi resulting in decreases in crop production, increasing mortality rates in animal species (Blehert *et al.*, 2009), and rise in infectious diseases which poses a serious threat to food security (Pennisi, 2010). More than 60% plants and over 72% animals are at risk of fungal infections (Fisher *et al*., 2012).

The introduction and dispersal of fungal pathogens is influenced by environmental and anthropogenic factors (Baiser *et al.*, 2012) which are pivotal for development of disease epidemics, and promote outbreaks of fungal infections (Aylor, 1990). According to Fisher *et al*. (2012), the ability of fungi to survive without their host as saprophytes or in spores form is a key factor to the success of pathogenic fungi spread. Climate and weather have been implicated in the introduction and wide spread of fungal pathogens (Anderson *et al.*, 2004), and wind which is a veritable vehicle of fungi dispersal. Disturbance of soil associated fungal pathogens result in dispersal of spores along with dust into air which travel great distances (Brown and Houmoller, 2002), while temperature and humidity directly influence the survival and spread of fungi. Fungi like other microbes are sensitive to weather changes (Harvell *et al.*, 2002). There is about 10-50% loss of perishable foods at the postharvest stages due to fungi infestation worldwide.

Fungi such as *Penicillium*, *Aspergillus*, *Botrytis*, *Fusarium* and *Rhizopus* species among others are associated with food spoilage (Mari *et al*., 2014; Barka, 2001), though grey, blue, green mould infections are common postharvest infections in fruits and vegetables caused by *Botrytis cinerea*, *Penicillium italicum*, and *Penicillium digitatum* respectively (Vitoratos *et al*., 2013). *B. cinerea* is an ubiquitous fungus of citrus fruits mostly found in wet and cold environment infecting fruits through injuries sustained by the fruits. It is one of the most studied necrotrophic fungal pathogen of plants (van Kan, 2006).

Control of post harvest fungal spoilage of fruits and vegetables is majorly done using chemical fungicides (Vitoratos *et al*., 2013); physical control method, natural antimicrobial and biocontrol fungicides have also been introduced as possible alternatives to chemical fungicides in order to produce safe and healthy foods (Gachango *et al*., 2012; Martinez-Romero *et al*., 2008, Usall *et al*., 2008). Another alternative to synthetic fungicide under consideration is the use of plant extracts in controlling the fungal rots of postharvest fruits and vegetables (Gatto *et al*., 2011). The use of microbial antagonist is gaining more attention and it is promising in disease management (Droby *et al*., 2002); bacteria (*Pseudomonas* and *Bacillus* species) and fungi (*Muscodoralbus*) are prevalent antagonistic microorganisms that have been used for post-harvest fungal disease control of fruits (Talibi *et al*., 2014; Lucon *et al*., 2010; Canamas *et al*., 2008; Verma *et al*., 2007; Meziane *et al*., 2006). Bio-save 110 and 100 are commercially produced bio-control bacterial antagonist of citrus postharvest rot infections that uses *Pseudomonas syringe* strain. Considering the challenge to develop strategies that are safe, environmentally friendly means of controlling postharvest diseases with limited or no risk to human and environment, the study aims to bring to the fore other bacteria sourced from the natural environment with potential to inhibit *Botrytis cinerea* potential at causing fruit infection, and thus leading to development of new ideas at controlling postharvest fungal spoilage.

Materials and methods

Sample Collection and Bacteria Isolation

Soil and water samples were collected into sterile sampling nylon and 50 mL Falcon tubes respectively and kept at room temperature. Water samples were collected from lochs in Heriot Watt University while soil samples were collected from both the University and Doctor Ruth Fowler's garden. One gram (1 g) of soil sample and 1 mL of the water sample were added into 9 mL of sterilized maximum recovering diluent (MRD) separately, mixed thoroughly using votexing machine. One millilitre of the resulting mixture were separately inoculated on nutrient agar plates and spread on the surface of the media using sterile spreader and incubated at 25^0C for 48 hr. Distinct colonies were sub-cultured overnight at the same temperature to obtain pure isolate of bacteria growth, and in nutrient broth for 24 hr at 25^0C on a shaker.

Characterization and identification of Isolated Bacteria species

Morphological, biochemical and nucleotide analysis method were used in the identification of the environmentally purified isolates. Biochemical screening were done after the Gram staining procedure was carried out, and isolates viewed under microscope to classify them on the basis of gram reaction and morphological appearances. Biochemical tests were carried out on the isolates using Microbact Identification kits (Microbact TM GNB12A/B/E, 24E) (Baron, 2001).

Cultivation of Botrytis cinerea

Pure strain of *B. cinerea* purchased from Leibinzinstitut (DSMZ-Deutsche Sammlung von Mikroor-ganismen und Zellkulturen GmbH) was added to 1 mL MRD and rehydrated for 30 min. The content was mixed gently using sterilized inoculating loop, 0.2 mL streaked on PDA plate and slant, and incubated at 24°C for 120 hr. The plates and slants of *B. cinerea* were kept in a confined jar to prevent dispersal of spores at temperature of 4°C.

Culture Preservation

Spores of *B. cinerea* were picked using sterile inoculating loop from 72 hr inoculated plate, transferred into sterile glycerol (80%), mixed gently and frozen at -80°C.

Screening for antifungal property of environmental isolates

The isolated bacteria species were tested against *B. cinerea* by the modified spot test procedure of Sindhu *et al*. (1999). Using sterile inoculating loop, colonies of the fungi were inoculated into 10 mL sterilized water, mixed by vortexing, and 0.1 mL of the mixture inoculated on the surface of PDA, and spread using sterile spreader. Four colonies from each pure culture of the isolated bacteria were inoculated into 1 mL sterile saline solution, and incubated for 48 hr at 25°C. Twenty microlitre (20 µL) of each isolate's mixture was separately spotted at specific point on the surface of prepared PDA plate containing *B. cinerea* properly labelled. Four isolates were spotted onto each plate of *B. cinerea*, incubated at 25°C for 120 hr; and the same procedure done on another PDA plate containing *B. cinerea* and 10 µL of each isolate. Bacteria with clear zones were separated, re-screened by repeating the process to confirm their antifungal activity. Those that maintained their antifungal activity were sub-cultured into 1 mL of nutrient broth for 24 hours at 37°C.

Results

Isolated Bacteria and their Biochemical and Molecular Characterization

Forty eight bacteria species were isolated from the eight environmental samples collected. Thirty five of these isolates were from soil samples while the other 13 isolates were from water samples. Of the 48 bacteria isolated, seventeen species belonging to the genera *Pseudomonas, Bacillus, Escherichia, Burkholderia, Staphylococcus, Streptococcus,* and *Proteus* had antifungal potentials were further identified while those with negative results were discarded (Table 1).

The seven bacteria genera with antifungal activities against *B. cinerea* were identified by traditional and molecular method. Base on Gram staining technique, isolate 2, 8, 12, 18 were Gram negative, cocci bacteria, isolate 15 and 16 were gram positive rod bacteria. Using identification kits, Microbact Identification kits (Microbact TM GNB12A/B/E, 24E), isolate 2 was confirmed to be *Pseudomonas stutzeri*, isolates 12 and 18 to be *Burkholderia pseudomallei* and *Burkholderia cepacia* respectively. Isolate 8 biochemical result was inconclusive, there was no perfect profile information marching the result in the data bank. Isolate 8 would have been categorized to belong to *Burkholderia* genus but it was oxidase negative and non-motile.

Antifungal Activities of Isolated Bacteria Species

Of the 17 isolates that showed positive antifungal potentials, only two completely inhibited mycelia and spores growth while seven others inhibited mycelia of the fungus with limited effect on the spores. The remaining eight isolates had little effect on both the mycelia and spores of the fungus, the inhibitions were apparent but not so distinct as shown in Fig. 1. *Pseudomonas stutzeri* and *Burkholderia cepacia* had the biggest zones average of 3.06 and 3.20 cm respectively, isolates *Burkholderia* sp., *Staphyloccocus aureus*, *Burkholderia pseudomallei* and *Streptococcus* sp were next with mean ranging from 2.41 to 2.90 cm while isolates *Bacillus subtilis*, *Pseudomonas aeruginosa, E. coli, Pseudomonas syringes, Pseudomonas stutzeri*, *Proteus mirabilis* and *Bacillus cereus* were with zones of inhibition ranging between 0.73 and 2.25 cm (Table 1).

Table 1. Isolates and their inhibition against *B. cinerea.*

Isolate Number	Isolated bacteria	Zone of inhibition (cm)
2	*Pseudomonas stutzeri*	3.06 ± 0.04
3	*Bacillus subtilis*	2.21 ± 0.02
4	*Pseudomonas aeruginosa*	2.25 ± 0.06
5	*Escherichia coli*	1.03 ± 0.03
8	*Burkholderia* spp	2.86 ± 0.03
9	*Staphylococcus aureus*	2.41 ± 0.02
12	*Burkholderia pseudomallei*	2.45 ± 0.03
15	Inconclusive	2.90 ± 0.02
16	Inconclusive	1.96 ± 0.02
17	*Pseudomonas syringes*	1.42 ± 0.02
18	*Burkholderia cepacia*	3.20 ± 0.02
19	*Pseudomonas stutzeri*	0.97 ± 0.03
20	*Streptococcus* spp	2.64 ± 0.02
22	*Burkhloderia cepacia*	0.73 ± 0.02
24	*Proteus mirabilis*	1.23 ± 0.02
25	*Bacillus cereus*	0.92 ± 0.02

Zones of inhibition were measured and recorded in centimeters as means ± standard deviation.

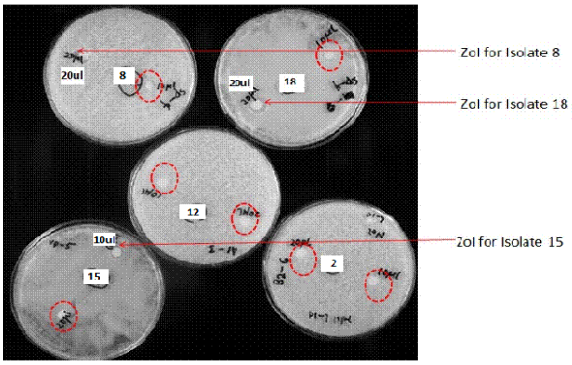

Fig. 1. Spot test results of antifungal activity of bacteria isolates 2, 8, 12, 15 and 18 against *B. cinerea.* Note: Isolate 8,15 and 18 produced zone of inhibition (ZoI), 1.03, 0.91 and 1.14 cm respectively on PDA (25°C, 120 hr) and 20 µl, isolate 2 and 12 produced no inhibitory activity on the *B. cinerea* on PDA (25°C, 120 hr). The inhibitory zones indicated with arrows on the plates and the red cycle indicating no zones.

Discussion

The present study is aimed at finding new biocontrol agent (s) against *B. cinerea*, a common pathogen that causes spoilage of fruits and vegetables. Several studies have confirmed the antifungal potentials of bacteria species against fruit and vegetables pathogenic fungi; with some plant growth promoting bacteria (PGPB) strains showing inhibitory effects on *B. cinerea* both in vivo and in vitro (Donmez *et al*., 2011). *Pseudomonas stutzeri* inhibition of *B*. *cinerea* with clear zone of inhibition was in agreement with earlier work presented by Donmez *et al*. (2011) though with different radii of inhibition.

Bryk *et al*. (2004) explained that the inhibitory capacity of *Pseudomonas* spp (B194 and B224) against *B. cinerea* spore germination and germ tube elongation in a liquid medium results from fragmentation and lysis of the fungus hyphae; and further explanation was given by Prasanna *et al*. (2014) who posited that *P. stutzeri* do not produce volatile metabolites that could inhibit fungi mycelia growth. Other researchers propo-sed that inhibitory activities of Pseudom-onas species against fungal pathogens is by cell wall degrading enzymes such as chitinases produced by the bacteria (Saima and Roohi, 2013). Other antifungal compounds have also been reportedly produced by some species of Pseudomonas which are also inhibitory to pathogenic fungi as reviewed by Jamalizadeh *et al*. (2011).

B. cepacia have been identified by researchers to be a known antifungal bacterium which supports findings in our report (Feki *et al*., 2011; Li *et al*., 2007; Quan *et al*., 2006). It is a complex bacterium with eight genomovars, of which some are pathogenic to plant and human while others are good biocontrol agents (Parke and Gurian Sherman, 2001). The antifungal activity of *B*. *cepacia* Cs5 in in-vitro and in-vivo studies on *B. cinerea* has been alluded to be the result of production of two analogous metabolites-Alkyl-Quinolones and Didecyl-Phthalate; however, other volatile and non-volatile compounds have been reported as contributing this inhibitory effects on *B. cinerea* (Kulakiotu *et al*., 2004).

Activity of *Bacillus subtilis* against the pathogenic fungi is probably due to the production of metabolites into the culture plate. *B. subtilis* have been identified to produce more than 20 volatile antifungal compounds capable of inhibiting germ tube elongation and spore germination of fungi in vitro (Arrebola *et al*., 2010). Identification of the microorganisms using PCR would have given the work a more precise knowledge of the microorganisms' identity, the primers were routinely used to amplify 1.465Kbp fragment of 16 Sr RNA which is highly conserved in bacteria species. PCR failed probably as a result of impurity in the extraction process or as a result of mis-labelled primer used. Growth factors such as temperature, pH, and nutrient content of the growth medium may also affect the activity of the isolates antifungal characteristic (Dalié *et al*,. 2010).

Conclusion

This research is in agreement with many other researches that have proven some bacteria possess antifungal characteristics. The result of this findings showed that *Pseudomonas* spp and *Burkholderia* spp have antagonistic capabilities against *B. cinerea*. However, more research is needed in identifying the compounds responsible for the antifungal activities in bacteria that showed positive potentials, understand the suitable condition for their activities, test individual compounds, and also synergize these compounds to see the effect on fungal pathogens and possibly process it for commercial use as alternative to synthetic fungicides.

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