Benha Journal of Applied Sciences (BJAS) **print: ISSN 2356–9751**
Vol. (7) Issue (4) (2022), (163-167) **online: ISSN 2356–976x**
http://bjas.journals.ekb.eg

screening of Antibacterial Activity of Different Secondary Metabolites from Streptomcyces Against Potato Brown Rot and Soft Rot Diseases

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Abstract
Streptomycetes consider as an important source of bioactive natural compounds. They are play an important role with tremendous applications in biological and biotechnological fields. Marine streptomycetes are particularly important microorganisms. Seven streptomycetes isolates were obtained from a marine source and tested for their ability to produce bioactive secondary metabolites. Their Antibacterial activity against were tested against a variety of potato pathogenic bacteria, including those that cause potato brown rot and soft rot illnesses. Using semi-selective media, *Ralstonia solanacearum* (a soil-borne pathogen) that cause potato brown rot was previously isolated from illnesses of potatoes with typical brown rot symptoms (SMSA). While the bacterium that causes potato soft rot (Pectobacterium atrosepticum) was isolated from infected potatoes and showed soft rot symptoms on logan's medium**.**

Keywords: Streptomycetes, secondary metabolites, antiacterial, potato brown rot and soft rot diseases.

1. Introduction

The (Solanum tuberosum) or potato is one of the world's important with a record production of 325 million tons in 2007 [11]. In Egypt, Potatoes are one of the most important horticultural export crops; however, the value of Egyptian potato exports decreased in 1995 from 102.12 million US$ to 7.7 million $US in 2000 due to the quarantine restrictions by the European Union on the potato brown rot, which used to account for 70-90 percent of Egyptian potato exports.

As a result, Egypt's Ministry of Agriculture's (Central Administration for Plant Quarantine CAPQ) has recently established a new Directorate for Internal Potato Quarantine to define pest free areas (PFAs), i.e. areas in which the causal agent of brown rot, (*Ralstonia solanacearum*), has not yet been detected [15].

Potato cultivation is gaining much interest due to its high value of vitamin C, protein, and potassium [27]. t's also used in the manufacturing of adhesives, binders, texturing agents, and possibly fuel-grade ethanol [3]. *S. tuberosum* is, unfortunately, severely infected with a variety of diseases. P. carotovorum and P. atrosepticum, formerly known as E. carotovora subsp. carotovora and E. carotovora subsp. atroseptica, respectively, are two pathogens that inflict substantial harm to potato yield and tuber quality [13]. The two main bacterial pathogens of potato, *Ralstonia solanacearum* and *Erwinia carotovora subsp.* carotovora (*Pectobacterium carotovorum subsp. carotovorum*), cause soft rot and brown rot and diseases not only in the field but also during storage. To keep these diseases from infecting potato crops, and to limit production loss, early detection of these bacterial pathogens is critical [22].

Microbes including (Bacteria, fungi) and plants have been considered as secondary metabolites produces that are not directly engaged in the normal growth and reproduction of the organism. Specialized metabolites, secondary products, and natural products are all terms used to describe them. Rather, they frequently mediate ecological interactions that can give an organism a selective advantage by increasing its survivability or fecundity. [25].

Streptomyces are Gram-positive bacteria with GC-rich, which belongs to Actinomycetales that are found all over the world and can be isolated from both marine (fish, corals, sediments, and sponges) and terrestrial (insects, soil, animals, and plants) habitats. These bacteria continue attract scientists interest for their ability to produce biologically active natural products that could be utilized in human medical application such as antimicrobial, immunosuppressants, anticancer agents [12]. Because of the emergence of antibiotic resistance, Streptomyces produces a structurally diverse antibiotics, which are critical in agriculture and medicine applications [21].

Streptomyces produces many of the world's antibiotics, making it an extremely significant bacterial group [20]. They can be found everywhere, ranging from high mountains to the deep sea [23]. They are Member of the phylum Actinobacteria, and are spore-forming bacteria. Streptomyces are non- motile microbes that produce hyphae that penetrate surfaces to find nutrition. They also produces aerial hyphae which divide to from spores that can withstand unfavorable.

conditions and are easily transferred to other locations to find new nutrient sources when resources are

few [4]. Streptomyces produces natural products during this phase, and these metabolites aren't basic for reproduction or growth but it play an essential role for giving the microorganism a competitive characters [4]. These compounds help vegetative bacterial cells by sequestering metals such as iron (siderophores), protecting them from UV light (through pigmentation), and suppressing competition (antibiotics), and allowing them to communicate with other species. Streptomyces' have a big genome, which can be quadruple the size of certain other bacterial genomes, allows for this molecular diversity. [2].

2. Material and Methods

2.1. Bacterial cultures and growth conditions for different isolates In this work, two extremely virulent R.solanacearum Phylotype IIa, sequevar 1 (race 3, biovar 2) strains, K3 and K10, were utilized. They were isolated in Egypt from symptomatic potato tubers with a clear classic brown rot symptom (brownish browning of vascular bundles with slimy whitish bacterialooze), and were previously recognized by the Bacterial Diseases Research Department in Giza, Egypt [8, 9].

Three virulent strains of P. atrosepticum, P.Carotovorum, and P. brasiliense were used in this study as a result of a pilot survey carried out in a previous study by [9] , which investigated potato plants/tubers with a typical symptom of soft rot and blackleg diseases (wet, slimy, black rot lesion spreading on stems and rotting tuber) in different locations in Egypt. The activity of several Streptomyces secondary metabolites was assessed using the 100-GH and MH3c strains.

For testing of bacteria (short-term usage), all bacterial strains utilized in this work were continually sub-cultured on nutrient agar and King's medium slants (18) , while lengthy preservation was done at -30oC using sterilized glycerol buffer (20%) [14].

Molecular identification was used for all isolates of R.solanacearum, P.atrosepticum, P.carotovorum and P. brasiliense using specific primers following [19, 17, 6], respectively .

In this context, bacterial DNA was obtained via extraction using following the method by [24] from pure bacterial cultures grown on NA media at 25°C. Specific primers (Rs-1-F: GCA TGC CTT ACA CAT GCA AGT C and Rs-II –R: GGC ACG TTC CGA TGT ATT ACT CA), (Expcc-F:GAACTTCGCACCGCCGACCTTCTA and Expcc-R: GCCGTAATTGCCTACCTGCTTAAG) and (ECA-1F: CGGCATCATAAAAACACG and ECA1- R: GCACACTTCATCCAGCGA) and (BR1f: GCG TGC CGG GTT TAT GCA CT L1r: CAA GGC ATC CAC CGT) were used for confirming P. carotovorum, P. atrosepticum, R. solanacearum, and P.brasiliense isolates, respectively.

For routine PCR experiments, all primers were bought from Bio Basic, Canada. Thermal cyclers were used to run amplification programme cycles (Applied Biosystems, UK). The following were used to make 25 µl of PCR reaction mixture: 5 µl of premade PCR master mix (Bioline, Germany's My Taq-red DNA polymerase), 1 µl forward primer (10 M), 1 5 µl reverse primer (10 M), 3 5 µl template DNA, and 17 5 µl ultra-pure water PCR products were separated on a 1.5 percent agarose gel and stained in TAE buffer with Red Safe (Intron, Korea) or ethidium bromide (Biorad, UK). In each loaded gel, a DNA ladder (1kb plus, Invitrogen, Thermo Fisher Scientific, USA) was utilized as a reference in at least one well. Gel was run on 80 V voltages at 400 mA (8 v/1cm). PCR bands were visualized via UV trans-illumination (at 355 nm).

2.2 Collection of marine samples and isolation of Streptomyces Samples were taken from a variety of marine sediment sources in the Red Sea. In 90 ml of sterilized saline solution (0.85 percent NaCl, w/v), samples (10 ml of overlying marine water) were transferred. Using serial dilution methods (10). After that, the prepared suspensions were inoculated in starch nitrate agar media for isolation of streptomycetes with the following composition (g/l): Starch 10.0, MgSO4. 7H2O 0.5, K2HPO4 1.0, NaCl

0.5, CaCO3 2.0, KNO3 2.0, FeSO4.7H2O 0.01,

Agar 20.0 and with different conc. of sea water (25, 50, 75 and 100%) were used in all the isolation media. After inoculation, The plates were incubated at 28° C and examined after 2-3 weeks for the growth of streptomycetes. Streptomycetes were identified based on their distinct morphological features (deep sitting colonies, characteristic colour, sporulation, etc.)

Pre-screening and antimicrobial activity Seven marine Streptomyces strains (S1 –S7) were cultured on rice-solid media at small scale (100 g rice soaked with 150 ml of 50 % natural sea water) at 30 °C for 7 days. Following incubation, each strain's culture medium was extracted via ethyl acetate, followed by decantation and filtration. Organic extracts were concentrated in vacuo before being administered to biological systems (antimicrobial activities against five bacteria).

Extracts (S1 – S7) were dissolved in CH2Cl2/10% MeOH at a concentration of 1 mg/ml. Aliquots of 40 µl were added on filter paper discs (6 mm) under sterilized conditions and dried for 1 h at room temperature. The paper discs were placed on inoculated nutrient agar plats medium (g/l): peptone, 10; Beef extract 3; and agar, 20. The pH was adjusted to 7.2 and the mixture was incubated at 37 °C for 24 hours. The disc diffusion test was carried out following the techniques of Bauer et al. (1966). The data was presented in the form of a mean standard deviation (SD). 2.3Fractination of bioactive metabolites

The resulting extract was subjected to column chromatography on silica gel G254 eluted by a DCM:methanol gradient, and the drops at the bottom of the column were collected and fractions analysed by TLC. The antibacterial activity of the obtained fractions was evaluated for Prioritizing the most antibacterial active fractions, the most active fraction was purified further using a Sephadex LH-20 column and Methanol as the mobile phase. The fraction with high antibacterial activity was purified further using preparative TLC based on the bioactivity of the resulting fractions from the sephadex LH-20 column. 3.Results and discussion

3.1Bacterial cultures and PCR confirmation

Using specified primers in a PCR experiment, all plant pathogenic bacterial strains employed in this investigation were confirmed. The isolates of R. solanacearum (K3 and K10) showed specific single band at 718bp. P. carotovorum isolates displayed a 550bp unique band. Furthermore, single bands at 690 and 322 bp were found in P. atrosepticum and P. brasiliense isolates, respectively.

According to [19] , the isolates of R. solanacearum (K3 and K10) displayed a specific single band at 718bp in the PCR experiment used to authenticate all plant pathogenic bacterial strains utilised in this investigation. According to [17]. The P. carotovorum isolate was verified using a particular band at 550bp. Furthermore, P. atrosepticum and P. brasiliense isolates were confirmed and revealed unique bands at 690 and 322 bp, respectively, as described by [6]. 3.2Isolation of streptomyces

A major motivation for discovering novel marine secondary metabolites is to avoid the problem of diseases that are resistant to currently available treatments [7]. Marine microorganisms are a rich source of new genes, which will almost certainly lead to the discovery of new drugs and targets. Sithranga is a medicinal product made from secondary metabolites produced by marine microorganisms [28]. So, based on their unique morphological traits, seven streptomyces isolates (S1-S7) were identified from the Red Sea (deep sitting colonies, sporulation, characteristic colour, etc.)

3.3Pre-screening study and antimicrobial activity

The paper-disk diffusion assay was used to pre-screen biological antibacterial activity of these isolates' ethyl acetate crude extracts against five distinct bacteria. Figure 1 and Table 1 While the bioactive metabolites of S7 were fractionated using various procedures, yielding nine distinct fractions. The antibacterial activity of these fractions was tested against five distinct microorganisms. The bactericidal activity of fractions [4, 5 and 6] is shown in Table 2.

Table (1) The crude extracts antimicrobial activity of streptomyces isolates (clear zone in mm.

Plant Pathogenic bacteria	Extract (1)	Extract (5)	Extract (6)	Extract (15)	Extract (16)	Extract (17)	Extract (18)
P. atrosepticum (MH3C)	9mm	7 mm	-ve	8 mm	6 mm	9 mm	9 mm
P. carotovorum (100GH)	7 mm	-ve	7 mm	9 mm	8 mm	10 mm	7 mm
R. solanacearum (K3)	8 mm	7 mm	7 mm	7 mm	8 mm	9 mm	10 mm
R. solanacearum (K10)	8 mm	7 mm	7 mm	7 mm	8 mm	9 mm	10 mm
P. brasiliense (BR1)	9 mm	9 mm	-ve	-ve	-ve	10 mm	10 mm
P. atrosepticum (Fel2)	9 mm	9 mm	8 mm	8 mm	9 mm	10 mm	11 mm

Table (2) The fractions antimicrobial activity of S7 (clear zone in mm).

Pathogenic bacteria/ Fractional streptomyces extracts	1	2	3	4	5	6	7	8	9
P. brasiliense (BR1)	12	12	11	12	14	13	12	-ve	-ve
R. solanacearum (K10)	11	12	13	12	13	13	11	11	-ve
P. atrosepticum (Fel2)	11	10	13	12	15	14	13	-ve	11
P. carotovorum (100GH)	-ve	12	11	14	12	11	12	11	-ve

Fig (1). Inhibition zone caused by crude extract of different Streptomyces spp. against P. carotovorum (100GH), the causal agent of Soft Rot disease in potato

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