

Recombinant Protein for Biocontrol of Brown Rot Disease in Potato

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Abstract: In Egypt, potato has an important position among all vegetable crops. Potato crop is infected with the brown rot disease producing a major problem which caused by *Ralstonia solanacearum*. Soil samples were collected from Gharbia governorate, bacterial isolation was carried out using suitable media. Many bacterial isolates were obtained and used for bioagent against *R. solanacearum*. One isolate among all the examined isolate showed high antagonism activity against the *R. solanacearum*. This isolate was subjected to identification using the 16S rRNA gene and the sequence analysis revealed that the strain is *Pantoea agglomerance*. The bacterium was cultivated on PBG medium and the culture filtrate was fractionated, the entire fractions were used for the antagonism activity. One of these fractions showed high ability to control *R. solanacearum* (named Biocine). The Molecular weight of the purified Biocine was determined by SDS polyacrylamide gel electrophoresis revealing one band with a molecular weight of 29kDa. Differential display was used to study the Biocine regulating gene. Band in molecular weight 800bp were excised, cloned using TOPO TA cloning kit. The fragment was released using the *EcoRI* restriction enzyme and subcloned into prokaryotic expression vector (Ptrac A). The recombinant bacteria were induced using the IPTG and the purified protein was obtained based on the His-tag technology. Bioassay was carried out for the purified recombinant protein compared with the wild one, the same activity was shown. It could be concluded that production a high amount of biocine on semi-industrial scale available. To transfer the experiment from lab to the field are under study.

Key words: Biocine • Brown rot disease • *R.solanacearum* • Differential display • Gene cloning.

INTRODUCTION

Economic Important of Potato Crop: Potato is one of the most important vegetable crops in Egypt, where about 20% of total area devoted for vegetable production is cultivated with potato. In addition, total cultivation of potatoes reached 197.250 faddan which produced 2,039,350 tons of tubers with an average yield of 10.34 tons / faddan [1]. Economic importance of this crop in Egypt attributed directly to its production affects severely its local and more importantly export impact, during their three seasonal plantations e.g. (Summer, Nili and Winter).

Potato plants are subjected to numerous of pathogens and insect pests which cause considerable loss in quantitative and qualitative potato yield. Such pathogenic and insect problems include the fungal pathogens and their diseases; *Alternaria solani* (early blight) and *Phytophthora infestans* (late blight); bacterial pathogens: *Ralstonia (Pseudomonas) solanacearum* (brown rot or bacterial wilt), *Erwinia carotovora* sub sp.

atroseptica (black leg and Erwinia rot), *Clavibacter michiganense* sub sp. *sepedonicum* (ring rot) and *Streptomyces scabies* (common scab) [2].

In Egypt brown rot disease was first recorded on potato, by Britton-Jones [3]. However, the bacterium was isolated for the first time in Egypt from potato tubers showing brown rot symptoms by Sabet [4]. In Germany, Gehring [5] isolated *Pseudomonas solanacearum* (Smith) from imported Egyptian potato tubers showing brown discoloration of the vascular ring. The pathogen nomenclature was changed successively from time to time ended by *Ralstonia solanacearum* named by Yabuuchi *et al.* [6] who suggested the name to the causal organism instead of *P. solanacearum*. Full description of this disease on potato and tomato was given by Kelman [7]. *R. solanacearum* is a strictly aerobic, non-spore forming, Gram-negative organism, with a wide and diverse host range affecting several hundred plant species from different 44 families, including the Solanaceae, Compositae and Leguminosae. Host plants of economic importance include potato, tomato, tobacco,

pepper, eggplant, groundnut and banana [8]. In addition, several ornamental plants and weeds can act as host reservoirs of infection. *R. solanacearum* is very complex and highly variable strains, *R. solanacearum* are grouped into five races according to the host or hosts primarily affected and five biovars according to the biochemical properties [9].

The most effective means of spread of brown rot (*Ralstonia solanacearum*) world wide is through distribution and planting of infected seed potatoes. *R. solanacearum* infected plant material has always been regarded as the greatest risk for both short and long distance. Although seed transmission has occurred in some European countries, all UK potato cases were due to water-logging following irrigation, or flooding from contaminated watercourses [10]. It is likely that contamination of water courses has occurred in the past through discharge of untreated waste from imported infected ware potatoes. The bacterium can also survive from season to season in potato ground keepers (unharvested potatoes from the previous crop) [11].

The survival of *R. solanacearum* (race 3 biovar 2) in the environment is not well understood. However, protecting the organism from desiccation and antagonism by other microorganisms can prolong this survival. *R. solanacearum* tends to persist longer in wet but well-drained soil, in the deeper soil layers (>75 cm), or in the presence of alternative crops, weed hosts or ground keepers. Soil survival is reduced by extreme cold and the presence of antagonistic microorganisms [10]. Infection caused by *R. solanacearum* for alternative hosts including solanaceous weeds, which increases the likelihood of survival and spread. Although, disease symptoms may not develop in these hosts. Bacteria continue to multiply and may become a source for re-infection of potatoes. Race 3 has been shown to survive and multiply in the roots of the secondary hosts Bittersweet (*Solanum dulcamara*) and Deadly Nightshade (*Solanum nigrum*) growing in rivers in a number of European countries [2, 10, 12].

Bacterial plant diseases are controlled by various methods, *i.e.* using resistant varieties, crop rotation, alteration of cultural practices and selection of free disease planting parts or seeds. In addition, using chemicals and antibiotics became a routine practice to control plant diseases which showed by time, little effect against majority of bacterial plant pathogens [13], as well as biological control has regarded as a promising alternative strategy to be used in the integrated control for plant diseases [14]. Among the biological control

methods, bacterial control agents can be either naturally occurring organisms (selected from the environment) or genetically engineered strains [15]. For effective biological control, the bacterial antagonist must be able to survive and grow under natural field conditions, where it can successfully compete on a long-term basis with the phytopathogens. Bacteria shown to have potential biocontrol action occur in many genera, *i.e.* *Erwinia* spp., *Pseudomonas fluorescens*, *P. syringae* and other biocontrol agents are not limited to a specific bacterial group. However, given the diversity of the *rhizosphere microflora*, it is probable that the full spectrum of potentially effective strains has barely been explored [16]. The aim of the present study was designed to look for a research new component in soil born, bacterium which could be able to control such pathogen.

MATERIALS AND METHODS

Indicator Strain: An indicator strain (SO₂) of *Ralstonia solanacearum* kindly provided by Prof. Nabil Sobhy Farag, Department of Plant Pathology, Agriculture Research Centre (ARC), Giza, Egypt was used in this study.

Isolation of Bacteriocin Producing Bacteria: Bacteriocin producing bacteria were isolated from soil samples that were collected from different potato infected fields located at Gharbia governorate, Delta region of Egypt. Serial dilution of 1 g soil sample in 100 ml sterile saline 8.5% NaCl were carried out according to One hundred μ l from the last dilutions (10^{-5} , 10^{-6} , 10^{-7}) was separately transferred under aseptic condition onto the surface of sterile Beef peptone glucose agar (BPGA) plates, then The inoculums was then spread using sterile glass spreader and left dry at room temperature for 10 min, then they inverted and incubated at 30°C for 24-48 hr. The single separated colonies appeared on BPGA plates were individually tested against the indicator strain using the toothpick technique [9].

Medium for Selection and Purification: Beef peptone glucose agar (BPGA) medium was used for growing the bacteriocin-producing bacteria or to maintain them for short and long term purpose. It contains 3 g beef extract, 5 g peptone, supplemented with 2 % glucose and 20 g / l Agar 1000 ml distilled water. The medium was adjusted to pH 7.2, then autoclaved at 121°C for 20 min without glucose, which was separately added to the medium after being autoclaved at 110°C for 10 min.

Ammonium Sulphate Precipitation: The cell free supernatant was then precipitated with 85% ammonium sulphate saturation by adding the solid salt slowly with gentle constant stirring in an ice bath. The pellet washed many times by buffer sulphate PH.7.0 using dialysis bag. The supernatant was dialyzed then protein content, bacteriocin activity and SDS- PAGE were carried out.

Determination of Protein by Lowery Methods: The protein concentration was determined in cell free supernatant and in precipitate after dialysis according to Lowery *et al.* [17] using bovine serum albumin standard.

Determination of Bacteriocin Molecular Weight Using Sds-page: Gel electrophoresis method described by Laemmli, [18] was followed. Samples were prepared by mixing small volume of bacteriocin sample containing about 1 mg/ml protein with (X2) application buffer; 0.125M Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercapto ethanol, 10% glycerol and 0.02% bromophenol blue; and then exposed to 100°C in water bath for 1 min. Each sample was applied to a separate well in the slab gel along with a pertained SDS molecular weight marker (14-205 K Daltons).

Electrophoresis was carried out at constant current 25 mA for about 1.5 hr. The gel was stained with comassie blue; 0.06% comassie Brilliant blue R-250 in 50% methanol and 10% acetic acid. The gel was distained overnight in a mixture of 60 ml methanol, 40 ml acetic acid and 800 ml distilled water.

Identification of Bacterial Isolate Biologically and Molecular: Bacterial isolate (S2HA) proved to have the ability to inhibit the indicator strain was preliminary identified according to morphological examination of bacterial colony, cell shape using light and electron microscopic examination, in addition to Gram and acid fast staining. Utilization of citrate, catalase, oxidase and nitrate were also performed [9]. Confirmatory identification using 16s rRNA assay was carried out after preliminary identification [9].

Bacterial Identification Using 16s Rrna Gene Amplification: According to Hafez and El-Bestawy [19], Primers 350F and 350R, corresponding to the polymorphic region of *E. coli* 16S rRNA conserved gene sequence, forward, 5' AGG ACG TGC TCC AAC CGC A `3 and Reverse, 5' AAC TGG AGG AAG GTG GGG AT `3 [20] were used to amplify approximately the 350bp of the 16s

rRNA gene. The genomic DNA was subjected to PCR reaction with conditions; initial cycle with 95C for 5 minutes and 34 cycles of 95C for 1 minute, 47C for 1 minute and 72 for 1 minute, followed by extension cycle at 72 for 10 minutes. The PCR product was visualized on agarose gel 1% and photographed using gel documentation system.

According to Lewington *et al.* [21], about 800 bp of the bacteriocine gene and R1(5'-CATATTGTAAAT TACCAAGCAA-`3) and the band was excised from the agarose gel and purified using the gel extraction kit (promega).The purified DNA was cloned into Biolink PBM-TEasy TA cloning kit (Promega). The recombinant bacteria were screened not only by scoring the white and blue colonies, also measurement of the inhibition zone for the pathogenic bacteria *R. solanacearum*.

Bioassay: The agar well-cut diffusion technique depends on the diffusion of bacteriocin radically through the agar layer from circular cup cut out from the agar layer. In this method, sterile Petri dishes were poured with a deep BPG agar medium inoculated with 1% (v/v) of *R. solanacearum* growth (grown for 48 hr at 80 °C. Five wells were punched out from the deep agar medium using clean sterile cork borer (5 mm in diameter) [22].

RESULTS

Bacteria were isolated from soil samples collected from different potato infected plantations at the Gharbia governorate, Delta region of Egypt. The isolates was found produce a large and clear inhibition zone against *Ralstonia solanacearum*.

These isolates were selected and tested for their ability to produce the inhibitory substance extracellular in a liquid culture. Three of the tested isolates showed a positive inhibitory effect when their supernatants were tested against the indicator bacterium *Ralstonia solanacearum* (Fig. 1D).

Determination of the Molecular Weight of Biocine: The molecular weight of the purified biocine was determined by SDS -PAG. The molecular weight of the target band was calculated based on protein molecular marker and the relative mobility of the calculated molecular weight was found to be 30 KD (Fig. 1C).

Identification of the Isolated Bacterial Using 16s R Rna Sequence: The 350 BP PCR products of the polymorphic regions of the 16S r RNA gene (Figure 1A) were subjected

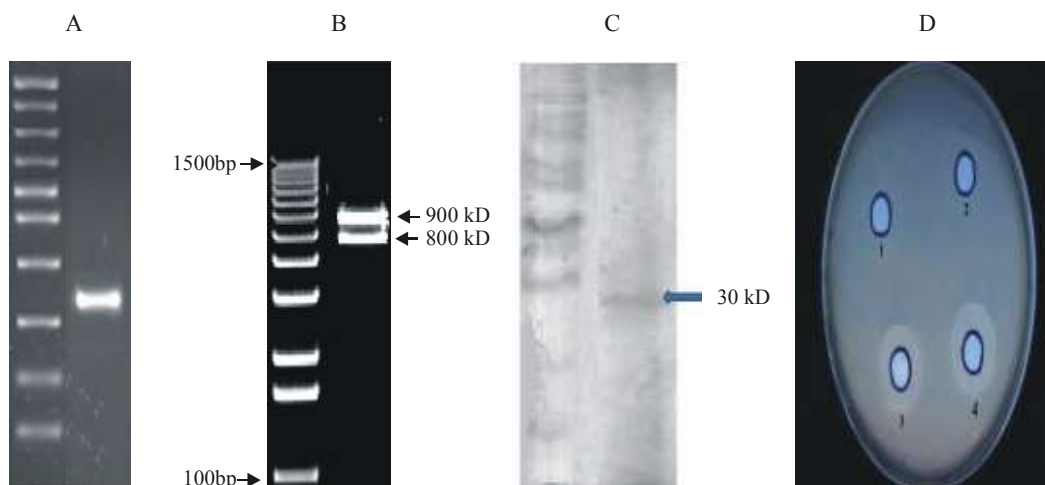


Fig. 1: A, B, C & D). A: 350bp of 16S r RNA gene. B: 800bp of the biocine control gene. C: SDS-PAGE of the biocine S2HA at 85% saturation ammonium sulfate precipitation level. Lane 1, protein pattern at 85% saturation ammonium sulfate; lane 2, protein pattern M, molecular mass standard. D: The inhibitory effect of Biocine activity against *R. solanacearum*.

to DNA sequencing (macrogen com. Korea). The obtained sequence was analyzed using BLAST DNA in: NCBI <http://www.ncbi.nlm.nih.gov/> the data analysis showed that the bacterial.

Isolate is *Pantoea agglomeranc* with similarity 88% with *Pantoea agglomerans* Strain xw112.

Cloning and Transformation of the Bacteriocine Gene:

About 800bp PCR product of the bacteriocine gene was cloned into the TA cloning kit and the productivity of recombinant bacteriocine were examined. The culture filtrate was spread on sterile Petri dishes containing a deep BPG agar medium inoculated with 1% (v/v) of *R. solanacearum*. The results shown in Fig. 3 revealed that there are different clear zones were obtained.

DISCUSSION

Antimicrobial compounds are of three main types; antibiotics, bacteriocins and siderophores. They are distinguished in terms of their chemical nature, antimicrobial activity and means of detection during *in vitro* culture. It cannot be inferred that bacteria will produce bacteriocin or siderophore without doing the appropriate test(s) and characterizing the antagonistic substance to prove its nature as a bacteriocin or siderophore [23, 24]. The chance of selecting effective strain may be improved initially by first isolating bacteria from the same environment in which they will be use [25]. In the present study, not only isolation and characterization of the antagonistic substance

produced by the some soil bacteria has been obtained but also a recombinant *E. coli* contains the bacteriocine gene was generated [26].

Isolation of many bacterial isolates and examine their ability for Bacteriocin was the main aim of this study. Soil samples were collected from contaminated areas with the bacterial brown rot pathogen and many isolates were obtained, subjected to morphological characterization. About ten isolates were used to examine their antagonistic ability against *R. solanacearum* and only one isolate showed the highest ability to inhibit such pathogen. The most promising isolate was selected and identified based on DNA sequencing for the 16S r RNA gene and the results revealed that the isolate is *Pantoea agglomeranc*. The results come in agree with El-Masry *et al.* [27] and Hechard and Sabl [28] who stated that Bacteriocin production has been shown to occur in various bacterial genera either gram negative or gram positive. Meanwhile, indicator strains are required to detect a particular bacteriocin [29]. Attempts to control bacterial plant diseases with bacteriocins have been started since 1972 [30]. It cannot infer that a bacterium will produce a bacteriocin without doing the appropriate tests including *in vitro* testes by looking for a zone of growth inhibition and characterization of the antagonistic substance to prove its nature a bacteriocin substance [31].

Precipitation of bacteriocin and to verify the effect of ammonium sulfate precipitation on protein content, SDS-PAGE was conducted. The present findings confirm the efficacy of ammonium sulfate as purification method

since it removed the majority of proteins from the bacteriocin containing solution. It is well known that the presence of one band in gel electrophoresis depends on the differences of the molecular mass for the separation of proteins (Fig. 1C). So, different proteins with the same molecular mass will appear as a single band in gel electrophoresis, these results come in agree with [32].

The bacteriocin controlling gene was isolated from these bacteria and cloned into TOPO TA cloning kit, sub cloning into Piracy expression vector. The recombinant protein was then used for controlling the pathogenic bacteria on the solid culture media. The results revealed that a high activity for the recombinant protein was demonstrated as shown in (Fig. 1D). Further studies are needed to look for new isolates might give us a new gene able to produce another substance able to control such pathogen.

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