Characterization of Actinomycetes Antagonist *Fusarium oxysporum* f.sp. *passiflora* Isolated from Rhizosphere Soil of Purple Passion Fruit Plants, South Sulawesi, Indonesia

Alimuddin Ali¹, Muhammad Junda¹, Herlina Rante², Riska Nuramelia³

¹ Laboratory of Microbiology, Department of Biology, Faculty of Mathematic and Natural Sciences, Universitas Negeri Makassar. South Selatan, 90222, Indonesia
² Laboratory of Pharmacy Microbiology, Faculty of Pharmacy, Hasanuddin University
³ Graduate School of Department of Biology, Faculty of Mathematic and Natural Sciences, Universitas Negeri

*muddin_69@unm.ac.id*

**Abstract.** To survey rhizosphere actinomycetes as potential biocontrol against fungal disease of passion fruits, rhizosphere soil of the plant were used as an isolation sources. Twenty five strains were assigned to Streptomyces-like strain based on morphological properties of spore chain. Four strain with distinguishing characteristic based on the macroscopic appearance of colonies on different media, were recovery from rhizosphere soil of passion fruits plant suggesting that various Streptomyces spp. grow surrounding of plant roots. On an agar medium, four strains (11.43%) commonly formed a clear growth-inhibition zone against fungal pathogen of passion fruits, *Fusarium oxysporum* f.sp. *radicalis passiflori* (FORP), indicating that this strains can produce antifungal substances. The present results indicate that four strain are a suitable candidate for the biocontrol of fusarium wilt.

1. **Introduction**

Multiple ecological interaction take place in nature; which can be negative or positive for the organism involved. Competition is an interaction encountered in all habitats since the organisms present need to do so in order to survive. Rhizosphere is one of the areas in soil where one can find abundance in microbial population. The high nutritional content promotes microbes to colonize in plant root area. However, the interaction that take place in the rhizosphere can be beneficial for the plant and also for the microbial community present.

Many types of microbial population such as bacteria and fungi have been found to be associated with the plant as endophytes or rhizospheric. The beneficial interactions of microbes with plants are being considered as an important area of research. Recently, many microbes have focused attention on feasibility of biological control for the plants pathogen such as fusarium crown and root rot and fusarium wilt. The disease results in severe loss of greenhouse and open field crops. Purple passion fruits (*Passiflora edulis* var. *edulis*) has become an important commercial crops of South Sulawesi for more than five decades. Passion fruit rust caused by the fungus has had incidences between 79 and 100% [5]. The increasing of production also means increasing use of agrochemicals such as fertilizer and pesticides. However, applying these agrochemicals in a long term can seriously threatening both local community and the environment.
Many recent report that microbes-based biocontrol can used as agent that could replace chemical control [7,8]. Actinomycetes especially Streptomyces spp. as excellent biocontrol agent to soilborne plant diseases, gives some beneficial for supplying numerous of nutrients, and plant growth promotion [9,10]. Soil bacteria belonging to the *Streptomyces* are regarded as promising biocontrol organisms due to their potential to produce a vast array of secondary substances such as enzymes and enzyme inhibitors [11]. Furthermore, actinomycetes are microbes that are known has ability to colonize of roots and gives numerous benefits both direct or indirect to plants such as improved growth [12], sideropore producers [13], as well as being able to producing biocontrol substances [14] and antifungal activity in rhizosphere soild sugar beet [15]. Therefore, it is important to explore rhizospheric actinomycetes from purple passion fruits and screened their potential in producing antimicrobial activities. The present study was carried out to characterizing the actinomycetes antifungi-producing from these unexplored soils rhizosphere of purple passion fruits plants. These strains were also characterized by molecular analysis based on nucleotide sequence generated from 16S rRNA region.

2. Experimental Details
The rhizosphere soils were collected from passion fruits plant at people farming garden in Malino, South Sulawesi, Indonesia. The samples were randomly collected within 200 to 700 m interval between the same site samples. Soil samples were obtained from a depth of 5 to 15 cm around of root and placed in sterile 50-ml conical tubes and stored at 4°C until isolation [16]. Isolation of strain was carried out immediately after samples were sent back to the laboratory.

Approximately 10 grams of each plant soils samples were transferred to 250 mL Erlenmeyer flask containing 90 mL of sterile destilated water. The suspension was shaken on 200 rpm for 30 minutes on rotary shaker. Further, the suspension was heated in water bath at 70°C for 1 hours and then allowed to settle for 15 minutes. Aliquots of 1 mL supernatant of soil mixtures were transferred to 9 mL of sterile destilalted water and subsequently to final dilution 10^6. About 0.1 mL of final dilution was spread plated on Starch Nitrate Agar plate [(20 g soluble starch, 0.5 g NaCl, 1 g KNO₃, 0.5 g K₂HPO₄, 3H₂O, 0.5 g MgSO₄.7H₂O, 0.01 g FeSO₄.7H₂O, 20 g agar, 1000 mL aquadest (pH 7.2-7.4), supplemented cyclohexamid 50 ppm]. Plates were incubated at 30°C for up 20 days. The pure of strains were preserved on 15% sterile glycerol suspension at -80°C for further investigation.

In vitro antifungal activities of rhizospheric actinomycetes isolates were assesed by using dual assay antagonistic method against the *Fusarium oxysporum* f. sp. *radicis-passiflorae* (FORP) [7]. Each of actinomycetes isolated obtained from isolation process was spreaded onto SC agar medium and incubated 35°C for 7 days. A plug agar of isolates (6 mm in diameter) was tranferred onto the edgen of Sabaroud Agar plate, while the tested fungi inoculated at the edge of the 9 cm plate on the same media. The plates were incubated 30°C for 7 days. Tested fungal plugs were also placed on uninoculated actinomycetes used as control. The endophytic actinomycetes was showed inhibition growth against to inhibited growth fungi considered as endophytic actinomycetes producing antifungal. The fungal inhibition was calculated from the equation:

$$R = \frac{(R_1 - R_2)}{R_1} \times 100$$

Where: \(R = \text{fungal inhibition (\%)}, R_1 = \text{the fungal growth radius of control culture}, R_2 = \text{the distance of fungal growth in the direction of actinomycete colony.}

Selected strain was grown on several media, namely yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts-starch agar (ISP4), (ISP7) were studied for culture characterized. The performance of aerial and substrate mycelia colour were determined by comparison with the Color Harmony Manual [18]. In order to examine morphological characteristics, the selected strains were grown on SC agar by using slide culture method and spore morphology were assessed by light microscopy. Physiological eahharacteristics of strains were determined as follow. Hydrolysis of starch was determined, utilization of carbohydrates and nitrogen as sole carbon and nitrogen sources was
tested by using medium (ISP9, respectively [19]. The temperature range for growth was determined on ISP2 in a temperature gradient incubator.

The DNA genome of strains extraction and purification was carried out using the Pure link genomic DNA kit (In-vitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. The 16S rRNA gene was amplified using the following primers following primers: 27f (5'-AGAGTTTGATCCTGCTGCTAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') to amplify 1500bp fragment. The PCR cycling was conducted as follow: pre-denaturation of the target DNA at 95°C for 3 min followed by 30 cycles at 95°C for 1 min, primer annealing at 55.5°C for 50sec, and primer extension at 72°C for 5 min, the reaction mixture was held at 72°C for 5 min. The resulting PCR products were visualized by electrophoresis in 1.5% (w/v) agarose gels stained with ethidium bromide [20]. The amplicon of DNA fragments was sequenced using sequencer model ABI 3100 sequencer according to manufacturers’ instructions (ABI PRISMA 3100 Genetic Analyzer User’s Manual).

The nucleotides obtained were subjected to BLAST analysis using by the NCBI database deposited in NCBI GenBank. The 16S rRNA gene sequence of strain was aligned with those of representatif strain members of other 39 Streptomyces genera using CLUSTAL-X. Phylogenetic trees were deduced by using the neighbor-joining method using the Phylip version 3.5 with bootstrap values based on 1000 replication.[21]

3. Result and Discussion

The thirty five of rhizospheric actinomycetes strain were successfully recovery on Starch Nitrate Agar from sites sampling of Passiflora edulis plantat people farming garden in Malino, South Sulawesi, Indonesia (Table 1). The passion fruit plantation area soil is a humus-rich soil with netral pH 6.6 to 6.7 so that it becomes a condition suitable for growth some bacteria such as actinomycetes. To avoid double strain, the colonies that showed the same morphological character were selected one colony as representative strain. A total of 4 strains were produce antifungal activity based on a dual assay (11.43%) of total strains. Morphological properties of strain were observed based on slide culture observation indicated that strain belonged to genus Streptomyces. The most important feature of Streptomyces are formation of powdery colony after incubated for 7 days, spiral spore chain. Order characteristics considered are mycelium branches and rare hypae fragmentation. Their mature colonies may contain two types of mycelia, substrate (vegetative) and aerial mycelia (Table 2).

| Strain groups number | Macroscopic morphological traits* | Antagonist against F.oxysporum (%) |
|----------------------|----------------------------------|-----------------------------------|
|                      | Aerial mycelium | Substrate mycelium               |                                  |
| # RML-A2             | Grey              | Brown                            | -                                 |
| # RML-A21            | White-grey        | Brown                            | -                                 |
| # RML-A21a           | White             | Brown                            | -                                 |
| # RML-A22            | Grey              | Brown                            | -                                 |
| # RML-A23a           | White             | White grey                       | 64.00                             |
| # RML-A24            | White             | Cream                            | -                                 |
| # RML-A24a           | White grey        | Blackish                         | -                                 |
| # RML-A25a           | White             | Cream                            | -                                 |
| # RML-A25b           | Grey              | Black                            | -                                 |
| # RML-A26            | Grey              | Cream                            | -                                 |
| # RML-A3             | White             | Yellowish                        | -                                 |
| # RML-A31            | Cream             | Red                              | -                                 |
| # RML-A31a           | Yellow            | Pink                             | -                                 |
| Strain groups number | Macroscopic morphological traits* | Antagonist against F. oxysporum (%) |
|---------------------|-----------------------------------|-----------------------------------|
|                     | Aerial mycelium | Substrate mycelium                |                                   |
| # RML-A31b          | Cream           | Black                             | -                                 |
| # RML-A32b          | Grey             | Black                             | -                                 |
| # RML-A32c          | White-yellow     | Black                             | -                                 |
| # RML-A32d          | Yellow           | Cream                             | -                                 |
| # RML-A41           | White            | Brown                             | -                                 |
| # RML-A42           | Cream            | Brown                             | -                                 |
| # RML-A42a          | Bright Grey      | Black                             | -                                 |
| # RML-B4            | White            | Grey                              | -                                 |
| # RML-B22           | Cream            | Grey                              | -                                 |
| # RML-B23           | Cream            | Black                             | -                                 |
| # RML-B33           | Grey             | Grey                              | -                                 |
| # RML-B41           | White-grey       | Brown                             | 67.54                             |
| # RML-B41a          | Cream            | Black                             | -                                 |
| # RML-B41b          | Grey             | Brown                             | 60.70                             |
| # RML-B42           | Grey             | Brown                             | 71.74                             |
| # RML-B42a          | Cream            | Pale pink                         | -                                 |
| # RML-B42b          | Cream            | Grey                              | -                                 |
| # RML-B42c          | Cream            | Brown                             | -                                 |
| # RML-B42d          | Grey             | Brown                             | -                                 |
| # RML-B42e          | White            | Grey                              | -                                 |
| # RML-B43a          | Grey             | Pink                              | -                                 |
| # RML-B43c          | Cream            | Brown                             | -                                 |

*SCA media - No antifungi activity

**Table 2.** Cultural characteristics of strain on different culture medium

| No | Agar media | Strain groups | Aerial Mycelium | Substrate mycelium | Soluble Pigment (SP) |
|----|------------|---------------|-----------------|--------------------|----------------------|
|    |            |               | HCM*            | Colour of colony   |                      |
|    |            |               |                 | HCM*               | Colour of colony     | Growth   |
| 1  | ISP1       | RML A23A      | RAL 7036        | Platinum grey      | RAL 1027             | Curry    | ++ | - |  |
|    |            | RML B41       | RAL 1013        | Oyster white       | RAL 1005             | Honey yellow | ++ | - |  |
|    |            | RML B41B      | RAL 9000        | cream              | RAL 1004             | Golden yellow | +  | - |  |
|    |            | RML B42       | RAL 9010        | Pure white         | RAL 1004             | Golden yellow | ++ | - |  |
| 2  | ISP3       | RML A23A      | RAL 7044        | Silk grey          | RAL 7004             | ++         | -  |   |  |
|    |            | RML B41       | RAL 9001        | Cream              | RAL 7032             | Stone grey  | +  | - |  |
|    |            | RML B41B      | RAL 9001        | Cream              | RAL 7032             | Stone grey  | ++ | - |  |
|    |            | RML B42       | RAL 9016        | Traffic white      | RAL 9010             | Pure white  | ++ | - |  |
|   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|
| 3 | ISP4 | RML A23A | RAL 7030 | Stone grey | RAL 1019 | Slate grey | ++ | - |
|   |   | RML B41 | RAL 9001 | Cream | RAL 1014 | ivory | + | - |
|   |   | RML B41B | RAL 9001 | Cream | RAL 1014 | ivory | ++ | - |
|   |   | RML B42 | RAL 1015 | Light ivory | RAL 1002 | Sand yellow | ++ | - |
| 4 | ISP5 | RML A23A | RAL 7030 | Stone grey | RAL 7032 | Stone grey | ++ | - |
|   |   | RML B41 | RAL 9010 | Pure white | RAL 1014 | ivory | ++ | - |
|   |   | RML B41B | RAL 1015 | Light ivory | RAL 3012 | Beige red | + | Brown |
|   |   | RML B42 | RAL 9010 | Pure white | RAL 9001 | Cream | ++ | - |
| 5 | BENNE TS | RML A23A | RAL 7030 | Stone grey | RAL 1000 | Green beige | ++ | - |
|   |   | RML B41 | RAL 1015 | Light ivory | RAL 3020 | Traffic red | + | - |
|   |   | RML B41B | RAL 3012 | Beige red | RAL 2002 | Vermillion | + | Brown |
|   |   | RML B42 | RAL 3015 | Light pink | RAL 3014 | Antique pink | ++ | - |
| 6 | TSA | RML A23A | RAL 9001 | Cream | RAL 1014 | Ivory | ++ | - |
|   |   | RML B41 | RAL 7032 | Pebble grey | RAL 1000 | Green beige | + | - |
|   |   | RML B41B | RAL 7032 | Pebble grey | RAL 1000 | Green beige | + | - |
|   |   | RML B42 | RAL 1014 | Ivory | RAL 1002 | Sand yellow | + | - |

*Harmony Colour Manual
++ : growth, well utilized ; +: growth, moderate utilized, -: no produce soluble pigment

Actinomycetes have been found most beneficial in rhizosphere for support the growth of plant host such as protect plant roots from pathogen and promote plant growth by producing antibiotic, hydrolytic enzyme or hormone substances [22]. The present study, genera of Streptomycetes are common genus that dominates the total population of actinomycetes in rhizosphere soil. This finding is in agreement with other reports which reported that actinomycetes belonging to genera streptomyces were frequently isolated from rhizosphere soil samples [23,24]. A total of 131 actinomycetes were recovery from rhizosphere and majority of Streptomyces-like strain [25]. Diversity of a community in a specific environment was dependent on the plants species since different crop varieties or species might produce different types of root exudates, which could support the activity of microbes [26,27]. Rhizosphere represents a unique biological niche that supports an abundance of diverse saprophytic microbes because of a high input of organic material derived from the plant roots and root exudates [28]. Distribution of genera actinomycetes influenced by several factor included humus content, pH of the soil and climate [29]. Actinomycetes are group of microbes with high abundance in the rhizosphere and approximately 70% are members of Streptomyces [30].

Evaluation of antifungal by antagonist dual assay approach indicated that actinomycetes obtained from Malino passion fruits plantation soil should have potential value as a source to find strain for protection of passion fruit plant which very susceptible pathogen fungi especially Fusarium wilt.
Actinomycetes strains were screened for antagonistic activity by measuring the inhibition zones present after 7 days of dual culture assay against mycelia growth of FORP. The highest antagonistic activity against FORP was observed in culture plate containing RML-B42 strain, in which a 71.74% inhibition zone formed due to antibiosis. Strain RML-B41 (67.54%) and RML-A23a (64%) are also shown inhibition zone, while the remaining strain RML-B41b generated. Four strains, produced powdery colonies and were capable of inhibiting the mycelial growth of passion fruit plant pathogenic fungi (FORP), in in vitro dual culture screening (Figure 1). These strains were identified as *Streptomyces* spp. according to cultural and morphological properties. Selected strain has a typical character as the *Streptomyces* genera. The strain showed the structure of spore chains opened spiral. Among the four culture media used, most of the strains growth was excellent in ISP media, whereas growth was moderate in Bennet’s and TSA media. This may be due to sufficient amount of nutrient included in this media was support for microbial activities. The ability of different Actinomycetes strain in utilizing various carbon sources was done by following the method recommended in International Streptomyces Project (ISP). Based on growth strain were compared with negative and positive control, it was observed that carbon sources was the most assimilated by all strain, except fructose and maltose not assimilated by strain RML-B41b and RML-B42, respectively. Furthermore, nitrogen sources assimilation showed that all strains not assimilated L-cystein. However, nitrogen compound most assimilated as nitrogen sources except L-asparagine and L-glutamate by strain RML-B41b and RML-B41, respectively. Almost all the strain have shown pH tolerances at different ranges of pH 6 to pH 8, and all strain shown good growth on pH 7 and 8. The interest result, all strain were most good growth on lower temperature (4°C). It is may be caused Malino distric was located in 2000 m above sea level. The strains were showed lower capacity to grow in 4% concentration of sodium chloride, so that it may be placed in the slightly halophilic organism. The microbes were grouped according to their requirement for NaCl concentrations i.e slightly (2% to 3%), moderate (5% to 20%) and extreme (higher than 12%) halophilic, respectively.

Characterization of actinomycetes strains were confirmed by the sequences of 16S rRNA that showed a single band to the expected molecular fragment size of the genes (±1500bp). The phylogenetic tree was divided into three main clades (trichotomy), known as I, II and III (Fig. 2). Clade I shows that all the strains demonstrated high similarities of 16S rRNA gene sequence to each other. All strain were well supported by the >50% bootstrap values, even the strain B high similarities compared to the reffered NR 042760. However, clade II and III show lower similarities of 16SrRNA gene sequences to each other that indicated by lower bootstrap values <50%. Based on the recorded data with regard to the physiological and biochemical properties of the strain, it is strongly confirmed that the all strains belonged to streptomyces genus. In order to confirm for the identification finding mentioned above, 16S rRNA gene sequenced obtained was subjected to GenBank BLAST search analyses. The phylogenetic tree shows that the strain was clustered in a clade of streptomyces spp.
**Figure 1.** *In vitro* inhibition of mycelial growth of *Fusarium oxysporum* f. sp. *radicis-passiflori* (FORP) of Actinomycetes strains by dual culture assay. Results are shown for: A. RML A23A; B. RML B41; C. RML B41B; D. RML B42, and E. Control. Ali et al.

**Figure 2.** Neighbour-joining phylogenetic tree inferred from 16S rRNA gene sequences of rhizosphere of passion fruits soils strain with related genera. Bootstrap values are expressed as percentages of 1000 replications. Score bar represents 1 nucleotide substitution per 100 nucleotides. Ali et al.

4. **Conclusion**
The study revealed the diversity of Streptomyces spp antifungal producing in rhizosphere of purple passion fruits plant. The selected strain described in the present study had a potency as the plant biocontrol of antifungal agent especially fusarium wilt disease.

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