MOLECULAR IDENTIFICATION AND MANAGEMENT OF RHIZOCOTONIA FRAGARIAE THE PATHOGEN OF BLACK ROOT ROT OF STRAWBERRY PLANT

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Received: Dec. 2016 / Accepted: May 2017 / Published: Jun. 2017 https://doi.org/10.25271/2017.5.2.364

ABSTRACT:
Strawberry plants are susceptible to a large number of pests and diseases and this can affect the quality and yield value of the fruit. Black root rot is an important disease of strawberry caused by a complex of fungi including Rhizoctonia. The most recognizable species of Rhizoctonia are R. solani and R. fragariae which are multinucleate and binucleate species, respectively. This work is aimed to isolate, identify and control the strawberry root rot caused by R. fragariae. Infected strawberry samples were collected from Erbil, Slemani, Duhok and Garmiyan Provinces. The identification of isolated fungi was achieved by using traditional methods along with molecular methods using polymerase chain reaction (PCR). In the later method, specific primers were designed and used to identify Rhizoctonia species. Several disease management options, including biological by using two species of Trichoderma, and chemical methods using Pristine fungicide, were also investigated. Sampling of strawberry plants revealed that the disease is prevalent in Kurdistan region and the isolated fungi, R. solani, Rhizoctonia sp., and R. fragariae, were pathogens of the disease causing crown and root rot of strawberry. PCR amplification was confirmed the identification of the species of Rhizoctonia. The results of control methods revealed that the most effective treatments were achieved using the fungicide followed by the use of the combination of T. harzianum and T. viride.

KEYWORDS: Strawberry, Rhizoctonia fragariae, black root rot, PCR.

1. INTRODUCTION
Strawberry Fragaria x ananassa Duch., the red delicious fruit, is one of the youngest domesticated plants. Nowadays, this plant is cultivated worldwide for its fruit which is rich in high nutrients like vitamin C, folate, and magnesium. It considered a very good antioxidant because it contains vitamin C and phenol compounds, and the potential role of strawberry consumption in human health and disease prevention is an active research area (Liston et al., 2014; Romandini et al., 2013; Giampieri et al., 2012). Strawberry is susceptible to a large number of pests and diseases, and this can affect on the quality and yield value of the fruit (Guerena and Born, 2007). The majority of these diseases are caused by such fungi as gray mold, powdery mildew, leaf spots, leaf blight, leaf scorch, black spot, fruit rot and anthracnose and a number of a soil borne diseases such as; Verticillium wilt, Fusarium wilt. The anthracnose, Black root rot, and crown rot are considered most common diseases of strawberry caused by Colletotrichum spp., Rhizoctonia spp., respectively. (Ullio, 2009, Ullio and Macarthur, 2011).

Strawberry black root rot is an important disease that caused by a complex of fungi include two most recognizable species of Rhizoctonia which are R. solani (the multinucleate) and R. fragariae (the binucleate) (Botha et al., 2003). In older seedlings, the fungus is limited to cortical tissues and elongate tan to reddish-brown lesions develop which increase to girdle the stem and may be twisted or die (Agrios, 2005). Rhizoctonia spp, the pathogen of root rots of strawberry considered to be a major problem that threat commercial strawberry production worldwide and classified based on their cell nuclear condition into uninucleate, binucleate and multinucleate (Fang et al., 2013).

There are several disease management measures have been employed to manage plant diseases. These include crop rotation, pathogen-free seed, removal of plant debris, and fungicide treatments. More recently, there has been an international attempt to the use of eco-friendly methods for controlling pests and diseases. Application of potential harmful chemical sprays are viewed with displeasure in many countries. Among these sound methods is the use of biological agents such as the use of the antagonistic fungus Trichoderma which have attracted the attention of their multipronged action against a wide range of plant pathogens (Harman et al., 2004).

Strawberry is a newly cultivated crop in Iraq, and the improvement of strawberry production and business in the country is done with the aid of U.S. Government through the USAID-Inma agribusiness program and by permeation of Iraq’s ministry of Agriculture in 2009. However, the cultivation of the crop is still restricted in small areas, research stations, house gardens and small farms (Bahram and Mohammed, 2006). Nevertheless, the importance of this crop is now increasing in Kurdistan, and few farms produce strawberry fruits and providing stocks to growers such as the one in Suse village/ Peramagron, in Slemani Province, comprising 10 greenhouses of strawberry which are growing a big red fruit variety Robygum continuously. Due to lack of any studies on strawberry diseases in Iraq in general and of Kurdistan region in particular, and due to growing demands on strawberry fruit and increase of new farms, this study was conducted and aimed to investigate the presence, identification and disease management options of black root rot pathogen.
2. MATERIALS AND METHODS

2.1 Sample Collection

Sampling achieved during December 2013 to November 2014 in several areas where different strawberry cultivars grown in Kurdistan region farms, these included Suse-Peramagron (Slemani Province), Kalar (Garmiyen Province), Gdrashara, Ankawa, and Haji Omran (Erbil Province) and Duhok and Akre (Duhok province). Infected plant parts were collected in strawberry fields, kept in cool boxes during their way to the laboratories.

2.2 Isolation

Infected plant parts washed under tap water for up to one hour to remove any dirt and soil on the outstanding plant surface, then were cut into 2 mm pieces, and then sterilised superficially by 70 % ethanol for 3 min, followed washing twice with sterilised distilled water to remove ethanol residues, then air-dried on sterilised filter paper before culturing on Petri plates containing fresh potato dextrose agar (PDA) medium and incubating at 25±2 °C for 2-5 days (Gilman, 1957 and Watanabe, 2010).

2.3 Identification

2.3.1 Traditional Identification: After the growth of fungal colonies, small pieces of young mycelium from edges of each colony was purified into new Petri plates containing PDA and incubated for one week. Fungal isolates were identified conventionally, using standard methods with the aid microscope and according to the taxonomic features described by (Watanabe, 2010).

2.3.2 Molecular Identification: Genomic DNA was extracted using DNA extraction Kit from Promega / USA based on the modified procedure from the same company. The isolation of genomic DNA was made directly on the infected strawberry samples, and from the fungi isolated. The following protocol was used to extract DNA:

1. Fresh mycelium (50 mg) of isolated fungi or the same amount of infected plant tissue were taken and frozen in liquid nitrogen and ground into a fine powder using a sterilized mortar and pestle.

   DNA was amplified using thermocycler (PCR machine) in 25µl (reaction volume) in PCR tubes using PCR master mix from Promega / USA. The master mix was composed of:

   • 12.5µl of 2x PCR master mix.
   • 1µl of each forward primer.
   • 1µl of each reverse primer.
   • 1µl of DNA template.

   The volume was completed to 25µl by adding nuclease free water.

   PCR was performed under the following conditions: initial preheat for 3 min at 95°C, followed by 35 cycles at 95°C for 15 seconds, annealing temperature were ranged between 62°C for 30 seconds followed by final amplification step 72°C for 15 min (Xiao and Ligard, 2004).

   Amplified DNA fragments were resolved on 1.5% Agarose gels (BIOMAX/Germany) for 60 minutes at 50 volts according to modified method of Sambrook and Russell (2001). The gel was prepared with 1 x TAE buffer and Ethidium bromide was added to provide a final concentration of 0.5 µg mL⁻¹. Four microliters of each PCR product was loaded in to the gel well with 4 µL of DNA size marker (100 bp ladder) (Promega, Madison, USA). A control sample (DNA-free sample) was also included in the gel electrophoreses. The gel was then visualised and using gel viewer (Mupid-2plas/Advance/Japan).

   Table 1. Primers used for detection of the pathogen of strawberry black root rot

| Primer name | Direction | Target fungus | Tm °C | Primer sequence | Source |
|-------------|-----------|---------------|-------|-----------------|--------|
| Rf1         | Forward   | Rhizoctonia fragariae | 62     | 5'-TGACATCTGCAAGACTCCA-3' | Designed with Primer 3 (GenBank: accession No. KP893156.1) (Zhong et al., 2016) |
| Rf2         | Reverse   | Rhizoctonia fragariae | 62     | 5'-AACACGGATCTTGGCTC-3' |        |

2.4 Pathogenicity tests

The pathogenic inoculum for R. fragariae was prepared according to the modified procedures of Xiangling et al., (2012) and Barbetti, (1989) as follows:

Fifty grams of millet seeds (Panicum miliaceum) were soaked in distilled water in 250 ml flask for 12 hours, the excess water was then poured off and the seeds were autoclaved three consecutive times each at 121°C for 20 minutes. The autoclaved millet seeds were sterilized using a biomass sterilizer and then moistened with distilled water and use 1 part of 1% agar solution, 1 part of 1% sucrose solution, 2 parts of the millet seeds, then poured into 250 ml Petri-dish and incubating at 25±2°C for 5 days.
in each flask were inoculated with 15 fungal plugs (5 mm) of each fungus taken from colonies grown on PDA. Flasks were incubated at (25±2) °C for two weeks and mixed gently every day to ensure equal colonisation. Strawberry seedlings were transferred to the 13 cm diameter pots containing a mixture of sterilized soil and peatmoss (2:1) inoculated with the fungus grown on millet seeds at a rate of 0.5% (w/w). The untreated control included growing seedlings in non-inoculated soil. The strawberry, cv. Sweet Charley and cv. Rubygum, which were produced and maintained by planting the stocks in a greenhouse at 25-30°C, were planted in the pots. All experiments were triplicated in Complete Randomized Design (CRD) with seven plants for each.

**Rhizoctonia** root rot disease was assessed on a 0 - 5 disease severity scale used by Fang et al., (2011) where: 0 = root well developed, no discoloration; 1 = <25% root discolored; 2 = ≥25%, <50% root discolored; 3 = ≥50%, <75% root discolored; 4 = ≥75% root discolored; 5 = all root discolored (rotted), plant death.

Crown disease caused by **Rhizoctonia** was assessed based on a 0 – 5 disease severity scale, where: 0 = no tissue discolored; 1 = <25% crown tissue discolored; 2 = ≥25%, <50% crown tissue discolored; 3 = ≥50%, <75% crown tissue discolored; 4 = ≥75% crown tissue discolored; 5 = all crown tissue discolored (rotted), plant death.

Disease symptoms of each individual plant were then assessed by calculating the proportion of stem discoloration on a 0-5 disease severity scale used by Fang et al., (2011) where: 0 = no crown tissue discolored; 1 = <25% crown tissue discolored; 2 = ≥25%, <50% crown tissue discolored; 3 = ≥50%, <75% crown tissue discolored; 4 = ≥75% crown tissue discolored; 5 = all vascular tissue discolored, plant dead.

The pathogenic inoculum was prepared according to previous procedure described by (Barbetti, 1989). The colonizing of *T. harzianum* and *T. viride* was performed by mixing 5 g of the commercial product of *Trichoderma*, Biocon-T (Ain Al-Sama, Saudi Arabia), with 5 kg of peatmoss, and incubated under greenhouse conditions at 25-27°C for two weeks. At the same time, seedlings of one-month old were brought and planted in 10 cm diameter pots. The experiment was contained five treatments in three replicates as shown in Table 2.

Table 2. Treatments used in control of strawberry black root rot caused by *Rhizoctonia fragariae*

| Treatment | Treatment descriptions |
|-----------|------------------------|
| 1         | Inoculating 15 strawberry seedlings by *R. fragariae*, colonized in millet seeds mixed with colonized peatmoss by *T. harzianum*, in the same rate that mentioned above. |
| 2         | Inoculating 15 strawberry seedlings by *R. fragariae* colonized in millet seeds mixed with colonized peatmoss with *T. viride* in the same rate. |
| 3         | Inoculating 15 strawberry seedlings with a millet seeds colonized by *R. fragariae* mix with a combination of equal amount of peatmoss that colonized with *T. harzianum* and *T. viride*, in the same rate. |
| 4         | Dipping the root of strawberry seedlings in Pristine fungicide before planting in peatmoss inoculated with colonized millet seeds by *R. fragariae* (dissolving 2g of fungicide in 250ml S.D.W). |

3. RESULTS

3.1 Isolation and microscopic identification

From the infected strawberry plant samples with visible symptoms brought to the laboratory from different locations, three species of *Rhizoctonia* were isolated from crown and roots of strawberry samples. Species of *Rhizoctonia* were isolated from all locations except Kalar (Garman Province). The direct isolation of pathogens from infected strawberry plants was achieved with the aid of cultural characteristics and microscopic features demonstrated that the most important suspected fungi were. *Rhizoctonia fragariae* and *Rhizoctonia solani*.

3.2 Molecular identification using PCR

To confirm the identification of *Rhizoctonia* spp., which isolated from discoloured crown and rotten roots, specific primers that detect the species of *Rhizoctonia fragariae* were used. Based on species- specific primers, the results revealed that four out of 8 samples screened were Binucleate *R. fragariae*. These include sample 4, 5, 6 and 8 with the band sizes of 250 bp (Figure 1). However, samples 2 (a suspected fungus described as a *Rhizoctonia* sp. microscopically), sample 3 (*Rhizoctonia* sp. isolate SS identified microscopically), and sample 7 (unknown species of *Rhizoctonia* having knobs and producing sclerotia from Suse) were not detected by the primers confirming that they are not *F. fragariae*.

![Figure 1. detection of Rhizoctonia species using polymerase chain reaction with specific primers.](image)

Table 3. Disease management (Biological and Chemical)

| Treatment | Treatment descriptions |
|-----------|------------------------|
| 5         | Fifteen strawberry seedlings inoculated with *R. fragariae* inoculum only by mixing colonized millet seeds with sterilized peatmoss only in a rate of 0.5% w/w (control). |

3.3 Pathogenicity tests of Rhizoctonia species

The results of pathogenicity tests for crown rot, root rot, and vascular tissues in Table 3 demonstrate that the highest and considerable pathogenic isolate for crown rot was *R. solani* (isolate SS2) with disease severity of 42.8% compared to the lowest one for *R. solani* (isolate Ho) with disease severity of 26.7%, and the virulence of Gr达尔asha isolate (Gr) and *R. fragariae* (SS1) were similar.

For root rot infections, the highest infection was by *R. solani* (SS2) with disease severity of 39.7% and the lowest one was also
The symptoms of *R. solani* (Gr.) as shown in (figure 2 A) was slightly rotten root with dark crown and pinkish vascular tissues. The symptoms appeared due to the infection by *R. solani* isolate (Ho) as in (figure 2 B) appeared as root discoloration with dark crown and vascular tissues. A reduction in root growth was occurred by *R. fragariae* (figure 2 C), the infected plants have brown to dark crowns and vascular tissues. In *R. solani* isolate (SS2) in (figure 2 D) infected roots was completely rotted and deteriorated with darkness crown and brown streaking of vascular tissues.

![Figure 2](image_url)

**Figure 2. Symptoms caused by Rhizoctonia spp. Isolates, where: A- R. solani (Gr) B- R. solani (Ho) C- R. fragariae (SS1) D- R. solani (SS2) and E- untreated control.**

### 3.4 Control of *R. fragariae*

A reduction of disease severity was occurred on strawberry plants when both species of the bioagent, *T. harzianum* and *T. viride* are applied, whether applied together or when used as individual treatments (Table 4). *T. harzianum* reduced the severity of wilted plants to 18.25%. However, *T. viride* was found to be more efficient when decreased wilting of the plants to 8.25% only. The effect of the combination of both *T. harzianum* and *T. viride* minimized infected plants to 16.5% only. The most effective treatment was the use of the fungicide, Pristine, when reduced plant wilting to 3.25% only.

| Treatment     | Root rot | Crown rot | Vascular tissue |
|---------------|----------|-----------|-----------------|
| *R. solani* isolate (Gr.) | 33.6     | 33.7      | 32.6            |
| *R. solani* isolate (Ho.) | 28.8     | 26.7      | 21.7            |
| *R. solani* isolate (SS2) | 39.7     | 42.8      | 40.5            |
| *R. fragariae* isolate (SS1) | 35.8     | 40.8      | 28.7            |
| Untreated control | 0        | 0         | 0               |

LSD (0.05) 0.80 0.61 0.85

### Table 3. Pathogenicity tests of isolates of *Rhizoctonia* species

The symptoms of *R. solani* (Ho) with 28.8% disease severities. The highest severity of 40.5% of vascular infection was caused by *R. solani* (SS2).

| Treatment     | Root rot | Crown rot | Vascular tissue |
|---------------|----------|-----------|-----------------|
| *R. solani* (Ho) |          |           |                 |
| *R. solani* (SS2) |          |           |                 |

The results of the study revealed that black root rot disease of strawberry is widespread in the Provinces of Southern region of Kurdistan. The major pathogen of the disease found to be three species of *Rhizoctonia*. Previous works by Fang et al., (2011) also confirmed that *Rhizoctonia* species are important pathogens associated with strawberry crown and root disease. In the current study, further confirmation of the pathogen was achieved by using PCR with specific primers was also proved the identification. This technique helps quick and accurate pathogen diagnosis among several pathogens associated with root rots. Previous work also concluded the identification using molecular methods and detected some 96 isolates of binucleate *R. fragariae* from diseased strawberry plants in Western Australia characterized by their nuclear condition, virulence, genetic diversity and polygenetic status (Fang et al., 2013). Results were also confirmed by Botha et al., (2003) that *Rhizoctonia* spp. and anastomosis groups were isolated from diseased strawberry plants in Western Cape Province of South Africa, both binucleate and multinucleate types were recovered from diseased roots and identified as *R. fragariae* and *R. solani*, respectively. The results of pathogenicity of *Rhizoctonia* isolates were investigated and found that *R. solani* isolates were most virulent causing severe disease symptoms on strawberry plants. Similar results obtained by Botha et al., (2003) in South Africa and found that *R. solani* isolates were the most virulent species causing sever stunting of plants. Nevertheless, *R. fragariae* sometimes also cause stunting in plant growth with small pale spreading lesions on the infected roots.

Two methods of control were evaluated to limit black root rot disease of strawberry. Although the use of the fungicide, Pristine, was the most effective method in minimizing the disease but the biological control using two species of the bioagent fungus, *T. harzianum* and *T. viride*, was also decreased the disease significantly. In a similar study to control *Rhizoctonia* carried out by Zhang et al., (2009) in which they used Bosalcalid fungicide (one of active ingredients of Pristine) to control isolates of *R. solani*, they stated that the fungicide’s efficiency was up to 58% in disease control. The greatest inhibition of *R. fragariae* pathogen on the symptomatic strawberry plants achieved when both *Trichoderma* species are combined in one treatment. Thus, the severity of crown and root rot and browning of vascular tissue resulted were 24%, 21% and 16%, respectively. Aly and

| Treatment     | % Disease Severity Index |
|---------------|--------------------------|
| Root rot    | Crown rot    | Browning of crown | Vascular tissue | Plant wilting |
| *T. harzianum* + *T. viride* | 24 21 16 16.5 |
| *T. viride*  | 35 30 19.6 8.25 |
| *T. harzianum* | 37.6 39 18 18.25 |
| Fungicide (Pristine) | 34.6 37.6 26.6 3.25 |
| Untreated control | 39.3 60 41 30 |

LSD (0.05) 35 32.25 42.75 9.25

### Table 4. The efficacy of different bio and chemical treatments on the severity of black root rot caused by *R. fragariae*

4. DISCUSSIONS
Manal (2009) also confirmed the efficiency of *T. viride* against *R. solani* agents in greenhouse. *Trichoderma* spp has been widely used as antagonistic fungal agents against several pests as well as plant growth enhancers (Verma et al., 2007). Disease severity of *R. solani* in daughter strawberry plants was reduced by 18-46% in treated nursery’s plots with *T. harzianum*. Furthermore, more isolates of *Trichoderma* spp. antagonistic to *R. solani* were found in infested field by the pathogen compared to non-infested one (Elad et al., 1981). Several modes of action have been proposed to explain the biocontrol of plant pathogens by *Trichoderma*, these include production of antibiotic and cell wall degrading enzymes, competition for key nutrients, parasitism, stimulation of plant defence mechanisms and combination of those possibilities (Harman, 2006). *Trichoderma* spp. generally grows in its natural habitat on plant root surface and therefore it controls root diseases in particular (Monte, 2001).

It can be concluded that strawberry plants are vulnerable to root rots caused by different species of *Rhizoctonia*. From these species, *R. fragariae* and *R. solani* are attacking strawberry plants widely. The results of this study revealed that early detection of root rot disease is possible with the use of PCR techniques and therefore, the disease could be managed properly. It could also be concluded from the results of this research that the use of systemic fungicides, such as the use of Pristine (a combination of Pyraclostrobin and Boscalid), is one of the best control methods to limit the use of mepronil and boscalid. It could also be concluded from the results of this research that strawberry plants were vulnerable to several pests as well as plant growth enhancers (Verma et al., 2007).

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نتایج مورد نظر نشان می‌دهد که مبتلایان و کنترل‌کننده ریزوکتونیا فراگاری Rhizoctonia fragariae هستند، به‌طوری‌که ارقام تعدادی از دو ناحیه در این مبتلایان افزایش یافته است. این مبتلایان نسبت به نوع خاصی از ریزوکتونیا، به‌طور شکلی گروه‌برداری شده‌اند. بیشترین تعداد ناشناخته‌های مبتلایان و کنترل‌کننده ریزوکتونیا در هر دو ناحیه به‌طور یکسان بوده است.

خلاصه بحث:

یک تحقیق در مورد بررسی ریزوفیت‌ها و کنترل‌کننده ریزوکتونیا فراگاری Rhizoctonia fragariae در کرکنی و بازار کردستان انجام شد. نتایج نشان داد که در این مبتلایان و کنترل‌کننده به‌طور کلی این نوع ریزوکتونیا موجود است.

در تحقیقات پیش‌گیرانه، نشان داده شده است که بررسی‌های بنیادی مبتلایان و کنترل‌کننده ریزوکتونیا در ناحیه‌های مختلف این دو ناحیه به‌طور کلی یکسان بوده است. بیشترین تعداد ناشناخته‌های مبتلایان و کنترل‌کننده ریزوکتونیا در هر دو ناحیه به‌طور یکسان بوده است.