Biological Control of *Fusarium oxysporum* Causing *Fusarium* wilt by *Bacillus subtilis* in Tomato

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ABSTRACT

Fusarium wilt is a fungal disease that attacks potato, tomato, eggplant and pepper. *Fusarium oxysporum* enter through the roots and interfere with the water conducting vessels of the plant. As the infection spreads up into the stems and leaves it restricts water flow, causing the foliage to wilt and turn yellow. The biological control is one of the most important goals of modern agriculture that reduces the possible side effects of chemical pesticides on human society. The objective of the present study was to isolate and identify *Bacillus subtilis* strains from the losses of date palm in order to biologically control the *Fusarium oxysporum*. The survey was done using 10 different soils sample from palm groves of Manoujan city, Kerman province, Iran. High potentials of isolated *Bacillus* sp. in culture media and solid state fermentation, high inhibitory effect of *Bacillus subtilis* on the *Fusarium oxysporum* were investigated. In tomato treatments, intensity, time and the types of wilt were compared. A total of 22 spore forming gram positive bacteria were isolated and identified. Tomato *Fusarium* wilt was observed in *Fusarium oxysporum* treatment earlier than other treatments. Using biochemical tests the isolates were identified among which isolate W7 was selected as the best isolate. In addition, *Bacillus subtilis* experienced a better growth on palm powders rather than crushed palms. According to the results, we can introduce *Bacillus subtilis* as a biocontrol to fight biologically against tomato fungal pests.

Keywords: Bacillus subtilis, Fusarium oxysporum, Biological control, Tomato

INTRODUCTION

Fusarium wilt (Fusarium disease) is a common vascular wilt fungal disease [1]. The fungus that causes *Fusarium* wilt is *Fusarium oxysporum* [2]. *Fusarium oxysporum* affects a wide variety of hosts such as tomato, tobacco, legumes, cucurbits, sweet potatoes and banana [3]. *Fusarium* wilt is one of the most important diseases of tomato in under cultivation lands of this crop which has been reported from five different continents [4]. *Fusarium* often exists in cotton and truck farming fields. But it can corrupt cotton or crops of vegetables. *Fusarium* grow as parasites on small and organic plants as saprophytic fungi and its spores are abundant in the air [5]. The symptoms of *Fusarium* wilt infection are wilting, chlorosis, necrosis, premature leaf drop, browning of the vascular system, stunting, and damping-off [6]. Tomato (*Lycopersicon esculentum* Mill) is an annual plant which belongs to Solanaceae family [7] which is one of the important products of Iran [8]. Thus, to increase yield and prevent damages, a great attention has been paid to the diseases of this plant, such as *Fusarium* diseases [9]. Fusarium crown and root rot of tomato are caused by the *Fusarium*

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oxysporum too. Fusarium crown and root rot of tomato impacts tomato production in Japan, Canada, Mexico, the United States of Ohio, Florida, California, New Hampshire, Pennsylvania, New York, New Jersey, and North Carolina [10]. One of the important agricultural plans is to replace pesticide with biological toxins, because pesticides have environmental side effects and impact the life cycles of soil directly and indirectly and at the end enter into the bodies of the living organisms through food [11]. Bacillus subtilis has been considered as biocontrol agent [11,12]. The bacteria rarely cause disease in humans and usually live as saprophytes in the soil which can be isolated from it [13]. Bacillus subtilis produce a series of lipopeptide antibiotics such as liturins that compete against other microorganisms, kill them or slow down their growth and also have a direct effect on plant pathogens [14,15]. It has been stated in the literature that the losses of the palm dates provide a suitable environment for the growth of fermentative bacteria as well as plants [16]. Some researchers reported that soil inoculation with Bacillus cereus, Bacillus subtilis and Trichoderma species, prevent seed infection caused by Fusarium species and also in this way, the antagonist activity of the root increases [17]. The goal of this study was to screen the native Bacillus subtilis strains from palm dates and their antagonistic activities on Fusarium oxysporum as biological control agent.

MATERIALS AND METHODS Soil sampling and isolation of *Bacillus* sp.

Sampling was drawn by sterile cellophane plastic and sterile steel castanets, of 10 regions of Manoujan city of Kerman province, Iran. 100 grams of each soil samples were collected from the surface and depths of 5 and 15 cm and placed in plastic containers. Samples were transferred to laboratory in cool boxes. The soil samples were diluted with normal saline (0.9%). 100 microliter from each dilution was inoculated on the surface of plate count agar medium (Merck Company) using spread glass. The plates were incubated at 30°C for 48 hours. The physical properties of the resulting colonies were analyzed. Gram staining and biochemical tests were conducted such as catalase, oxidase, Simmon citrate, motility, indole, gelatin liquefaction, sugar fermentation, and the ability to produce amylase [18]. Palm date losses (Mordasang type) were used. Three different samples including fresh palm dates, dried palm dates and dried powder of dates were selected to be used in the fermentation test in the solid state fermentation. In order to produce live biomass fermentation test was conducted. Fusarium oxysporum (c-1024) was prepared from agriculture organization of Iran. The fungi were inoculated on Sabouraud dextrose agar (Merck Company) and incubated in aerobic conditions for 7 days at 30°C. Then using macroscopic and microscopic diagnostic tests, the fungus was confirmed. Isolated bacteria were inoculated on plate count agar and incubated at 30°C for 24 hours. Then Fusarium oxysporum inoculated adjacent to the bacteria at a distance of 50 mm to evaluate the antagonistic properties of isolated *Bacillus subtilis*. To evaluate the effect of isolated bacteria in the screening stage on the prevention of pathogenic Fusarium oxysporum corruption of tomato, different treatments were used, which are described below. Tomato seeds after being sterilized by alcohol were placed in the sterilized soil of the pot. 20 seeds were put in each pot. Then the following treatments were received. The first treatment: the seeds of tomato were placed in three pots (three repetitions) as control samples. These seeds didn't receive any treatment. The second treatment: the tomato seeds with fungal spores were planted in the pot; 5 ml diluent containing fungal spores of the *Fusarium oxysporum* $(1.5 \times 10^5 \text{ spores/ml})$ were planted in three pots (three repetitions). The third treatment: tomato seeds with fungal spores (as stated in the previous method) with 5 ml bacteria suspension as in 1.5×10^8 /ml were planted in three pots (three repetitions). The forth treatment: the Fusarium oxysporum used in previous treatments was inoculated with the soil of the pot and 0.8 ml of a commercial fungicide called Mancozeb was added to the soil in three pots. After the growth of tomato seeds and the germination of the leaves, a

bacteria suspension of 1.5×10^8 bacteria/ml were sprayed on the surface of the leaves and stem. All samples were examined for 20 days in the ambient temperature (28-30°C) to study antagonistic effects of bacteria in the prevention of Fusarium wilt [13,19].

RESULTS AND DISCUSSION

According to the date palm losses cultivations, 22 different colonies of *Bacillus* sp. were isolated and purified. After reviewing the results of diagnostic and biochemical tests, 9 isolates were identified as Bacillus subtilis. Bacillus subtilis (isolate W) displayed the strongest inhibitory effect on the growth of the fungus. Bacillus subtilis were grown on the powdered dates after 48 hours. However, they did not grow on the crushed date. In the temperature of 30°, 7 mm date palms fermented most. Also, according to the results, the isolated Bacillus subtilis in the screening stage, is able to prevent from growth of *Fusarium oxysporum*, in other words, the plant does not display the pathologic signs including wilted leaves, curvature stems and dryness and thus can be utilized as a biological control agent. Yet, in pots inoculated by Fusarium fungus which lacked bacterial treatments, pathogenic symptoms (wilt, curvature and drying of leaves and stem) were observed. This showed the pathogenic abilities of the fungus. The tomatoes in the pots that did not contain any fungus and bacteria in comparison with the ones containing pathogenic fungus, displayed no symptoms of wilt and dryness, this fact proves the appropriate conditions of growth. Therefore, the symptoms such as wilt, curvature and dryness are related to pathogenicity of fungus and results are reliable. The wilt of the leaves was relatively higher in cultures containing anti-fungal toxin Mancozeb and Fusarium oxysporum in comparison to bacterial treatment samples. In addition, in comparison to samples not having the bacterial treatments, it showed a better performance. So the results of bacterial treatment achieved better performance in the control of Fusarium oxysporum.

Keeping ecological balance in the production of agriculture crops is the basis of sustainable agriculture. Unreasonable use of agricultural pesticides to control the damaging factors is one of the most important factors disturbing the natural balance in the production of healthy agriculture crops. The uncontrolled use of chemical pesticides may cause severe food poisoning and pollution. In addition, the resistance of pests to pesticides is another common problem caused by uncontrolled consumption. To reduce the negative effects of the use of pesticides, different strategies have been considered recently. One of them is biological control. Biological control of pests is a natural way to keep the population of pests in a low level which is active in all natural and unnatural (human made) ecosystems [20]. The outcome of biological control is having a healthy land in which the plants level of production is appropriate and satisfactory and the natural balance is preserved. Natural control of pests, having the objective of reducing the population of pests, has existed from the first ecosystems, 500 years ago and has occurred without human interference. Biological control of pests is a natural way to keep the population of pests in a low level which is active in all natural and unnatural (human made) ecosystems [21]. One of the advantages of biological control is the reduction of poisoning risks by toxic chemicals and the absence of pesticide residues on products. A choice for reducing pollution caused by the use of chemical and synthetic agrochemical in tomato disease management is biological control by using of antagonist bacteria belonging to the Bacillus genus, because they are considered the most efficient for their inhibitory properties [22]. Some investigations showed that there are different microorganisms used in Fusarium wilt control, such as Trichoderma species which through antibiosis and parasitism destroys the pathogenic fungus or reduces its effects [23]. In this study, we attempted to identify and isolate the Bacillus subtilis from the palm grove soils. Based on obtained results, Bacillus subtilis isolate W had the most antagonistic activity on growth of Fusarium oxysporum in both laboratory and in vivo conditions and in conclusion it can be effective biological control agent against plant pathogenic fungi such as Fusarium oxysporum.

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