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Fusarium oxysporum a possible agent for biological control of *Papaver somniferum* in the Middle East

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ABSTRACT

There is a great need to make biological control research applicable to local political and social situations. The objectives of this study were to evaluate the effect of *Fusarium oxysporum* isolates against opium poppy (*Papaver somniferum*) and verify their potential as biocontrol agent. For this, a 2-year experiment was carried out under glasshouse (2 trials) and field conditions (2 trials). From infected poppy plants, a total of 16 pathogenic fungal strains were identified as *F. oxysporum* and used for the experiments. The isolates Ghr18, Ghr5-2, Mr28 and Ghr5-4 caused the highest wilting symptoms on sample plants ($P < 0.001$). In addition, no significant differences were observed between field and glasshouse conditions ($P > 0.5$). Moreover, the results showed a clear host specificity of the selected pathogenic isolates. These results suggest that *Fusarium* isolates have the potential to be used as biological control agents against poppy plants in the Middle East.

1. Introduction

Despite many distinct political and governance tensions related to the attempts to control opium (*Papaver somniferum)* cultivation in the world, the production of opium poppy has increased in the last couple of years. Afghanistan remains the biggest producer of opium, with an estimated area of 224,000 ha under cultivation in 2014, a 7% increase from the previous year (UNODC, 2014). Not only Afghanistan suffers ravages of terror and insecurity as results of drug production, countries around its borders face security concerns with regards to drug trafficking issues. A sizable proportion of opium in Afghanistan is trafficked and cultivated illegally in Iran, as Iran shares 1923 km-long Eastern border with this country, and consequently sent on to consumer markets in Europe. Although Iran efforts to combat drug trafficking has been praised by United Nations and Interpol, as the country typically accounting for 74% of the world's opium seizures and 25% of the world's heroin and morphine seizures (http://bjc.oxfordjournals.org/content/53/2/179.full; Paoli et al., 2009; UNODC, 2014), the Afghanistan opium cultivation remains a major challenge for Iran. One of the major consequences, is the illegal poppy field cultivations and small-scale heroin productions in rugged hillsides of western boarders of Iran which are influenced by the cultivation patterns in Afghanistan.

There is a long history and successes in the biological control (biocontrol) of weeds, plant diseases and agricultural pests. Biocontrol procedures emphasize host-specificity testing to select highly specific candidate agents to reduce the risks associated to non-target species (Lutwick and Lutwick, 2009). Around the globe, most of the biocontrol programs schemes have mainly attacked plant pathogens, the main threat on crops; however, very limited studies have used plant pathogens as bioherbicides to control narcotics supply in the Middle East.

Fusarium species are one of the largest genera of fungi that cause various diseases such as crown rot, head blight, and scab on crops (Saremi and Saremi, 2013). Many studies have demonstrated the potential of non-pathogenic *F. oxysporum* in controlling various *Fusarium* diseases, including *Fusarium* wilt, based on actions of competition, mycoparasitism, antibiosis and induction of plant defense reactions (Cachinero et al., 2002; Larkin and Fravel, 1998; Lecomte et al., 2016; Mandeel and Baker, 1991; Minuto et al., 1997; Shishido et al., 2005). Strains of pathogenic *F. oxysporum* have also been selected as potential biological control agents and mycoherbicides to control and manage various parasitic weeds by destroying the tissues (Ndambi et al., 2011; Saremi and Okhovvat, 2008; Zarafi et al., 2014).

Besides the use of biocontrol approach against plant diseases and invasive weeds, numerous studies have demonstrated the potential use of fungal strains as biological control agents for various narcotic crops. O'Neill et al. (2000) reported *Dendryphion penicillatum* and *Pleospora papaveracea* as destructive seedborne pathogens to *P. somniferum* which caused complete poppy blight; however, *P. papaveracea* was more

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virulent and produced ascospores in addition to conidia, therefore has more potential for use as a mycoherbicide. Isolates of *Fusarium oxysporum* f. sp. *erythroxyli* showed significant effect on coca plants (*Erythroxylum coca* var. *coca*) death and high disease rates, when applied to soil in both greenhouse and field experiments (Bailey et al., 1996). Other studies have also used host specific *F. oxysporum* strains as an alternative to control coca (e.g. Bailey et al., 1997; Sands et al., 1997), hemp (*Cannabis sativa* L.) (e.g. Hildebrand and McCain, 1978; McCain and Noviello, 1985) and *P. somniferum* (Connick et al., 1998; McCarthy et al., 1995).

In recent years, elevated awareness of the use of mycoherbicides on other countries (agricultural bioterrorism) and the threat of simulation to cause direct damage in agricultural sector have resulted in efforts to reduce the biocontrol programs especially towards the narcotic plants (e.g. Suffert et al., 2009). Nevertheless, the benefits of no detrimental effect on human and animal health which present a low risk for environmental damage over man-made chemical solutions as well as the host specificity of *F. oxysporum* (Buxton, 2006), can suggest biocontrol approach as one of the strategies to reduce narcotics supply in particular at local and regional scales.

Despite the strong regulations to prevent illegal cultivation and production of narcotics crops by Iran authorities, opium poppy continues to be cultivated in remote areas near neighboring countries. The present study was initiated to isolate and identify strains of *F. oxysporum* from three different regions and to find out whether the isolates were efficient biocontrol agents for opium poppy. To the best of our knowledge, this is the first report on *P. somniferum* biocontrol that was carried out under both greenhouse (laboratory scale) and field conditions, in the Middle East. It should be noted that this study was conducted with the consent of the government authorities.

2. Materials and methods

2.1. Field survey

Field survey was carried out from June 2013 to July 2014 within three different locations[1] (hereafter referred as Ghr, Mr and Z) in Iran, close to boarders of Afghanistan. Opium plants (*Papaver somniferum* var. album) showing symptoms of chlorosis, foliar wilting and necrosis (traces of *F. oxysporum)*, and rhizosphere soil samples were collected from the three regions. The three locations were almost aligned along a straight line and the calculated distance between Ghr and Mr was 180 km and Mr to Z was 60 km. A total of 40 samples were collected from each site, overall 120 samples.

Opium poppy largely tends to be winter or spring crop (sown from October to February) and harvested between 120 and 250 days later depending on the variety and environmental conditions (Chouvy, 2011). The mean elevation, average temperature, average relative humidity and the annual rainfall of the study areas are approximately 800 m asl, 20 °C, 18% and 100 mm, respectively.

2.2. Isolation of Fusarium species

All of the plant samples were soaked in ethanol and 1% NaOCl and rinsed with sterile distilled water and later cultured in Peptone PCNB Agar (PPA) (Burgess et al., 2008). In order to isolate *Fusarium* species from soil samples the serial dilution technique was used, serial dilutions (10^{-2} to 10^{-4}) were prepared and plated onto PPA medium. After 5–7 days of incubation, the colonies were purified by hyphal tipping (Burgess et al., 2008; Saremi and Saremi, 2013). For the species to be identified at genus level, they were sub-cultured on water agar and after couple of days were mounted on microscope slides for further examination.

[1] Due to security restrictions, the names of the study areas are not exposed.

2.3. Identification of Fusarium species

Species identification was based on the morphological characteristics of single-spored isolates as described by Leslie and Summerell (2006). The *Fusarium* species were identified on the basis of macroscopic characteristics such as growth rate of the colony, pigmentation, absence or presence of microconidia, characteristic of macro- and microconidia and conidial measurement.

2.4. Molecular identification of F. oxysporum

Total genomic DNA of *F. oxysporum* was isolated using the method described by Raeder and Broda (1985). To generate molecular markers, the polymerase chain reaction (PCR) approach was used. For the molecular identification of *F. oxysporum* isolates, two primers designed specifically to the internal transcribed spacer (ITS) region of the rDNA operon of *F. oxysporum* were used (Mishra et al., 2003). All isolates were identified using *F. oxysporum* specific primers FOF1 (5′-ACA TAC CAC TTG TTG CCT CG-3′) and FOR1 (5′-CGC CAA TCA ATT TGA GGA ACG-3′). PCR reactions were carried out in 50 µl reaction mixture containing 5 µl of 10 × PCR buffer, 0.6 µl of MgCl2 (50 mM),1 µl of each dNTPs (10 mM), 2 U *Taq* DNA polymerase, 1.5 µl of each primers, 6 µl of DNA (10 ng) and 31.4 µl ddH2o. Amplification was performed with an Corbett DNA thermocycler (Corbett Research, Mortlake, Australia) and based on the method suggested by Mishra et al. (2003), in a program comprising of 34 cycles of the initial denaturation at 94 °C for 60 s, annealing at 53 °C for 60 s, and extension at 72 °C for 1.5 min with an initial denaturation of 5 min at 94 °C before cycling and final extension of 5 min at 72 °C after cycling. PCR products were electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining.

2.5. Glasshouse evaluation

Glasshouse experiments (2 trials) were performed based on the pathogenicity test results of selective isolates. In order to investigate the pathogenicity of *F. oxysporum* and identify the *forma specialis,* isolates were maintained in potato dextrose agar (PDA) slants and incubated for 7 day at 29–30 °C. The mycelial plugs (5 mm diameter) of the isolates were then positioned in sterile sand: maize meal medium (50 g + 1.5 g maize meal + 10 ml water) and incubated for 15 days at 28 ± 2 °C (for preservation, isolates were stored on silica gel at 4 °C (Windels et al., 1988)). Pots of 25 cm diameter were filled with mixtures of sand, soil and animal manure in the proportion of 4:2:1. For inoculation, before seeds were sown in the pots, the top 5 cm of soil was removed and mixed with 15 g of inoculum and distributed through the pots. The sterile seeds were then planted immediately in 1 cm depth and compost were added to the surface, and irrigated afterwards.

Additionally, single-spored isolates of *F. oxysporum* were sub-cultured onto PDA media and grown for 10 days at 20 °C. Spore suspensions were produced by adding sterile distilled water to the Petri dishes, gently removing spores using a glass spreader. The spore suspension concentration was adjusted to a concentration of 1×10^6 spore/ml using a hemocytometer. At the beginning of pod development (R3 stage), root surface and the adjacent tissues of the lower stem of the plants were inoculated with a drop (1 ml) of spore suspension. The observation on wilt incidence and symptoms were recorded at harvest approximately 110 days after sowing.

The glasshouse temperature was approximately 20 °C, and daylight was supplemented with light from fluorescent tubes to provide 14 h of continuous light. The experiment was set up in a completely randomized design with three replicates (pots) per isolate and three replicates for negative control pots (equivalent weight of maize meal media without the inoculated *Fusarium*).