WEED PATHOGENIC FUNGI FOR BIO-CONTROL, IMPROVED MANAGEMENT AND A SAFER ENVIRONMENT

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Abstract - The increasing ratio of herbicide-resistant crops and weeds in addition to serious public concern about herbicides harmful effects will continue to be the driving force for investment in, and investigation of biocontrol agents. Biological control is highly specific and represent an environmental friendly long term solution to control weed especially herbicide resistant ones. This study aims to highlight the importance of weed pathogenic fungi as a promising approach to control herbicide resistant weeds. Different fungal species were isolated from infected stems and inflorescences of juvenile bean broomrape (Orobanche crenata) and were evaluated as biocontrol agents. Culture filtrates activity on broomrape seeds as well as mixtures of active fungal conidia and mycelia were tested either in plastic bags or in plastic pots in presence of the host plant. Fifty-one fungal species were isolated and identified during this study (29 from infected stems and 22 from infected inflorescences) on diseased juvenile bean broomrape. The in vitro assay on the germination of the parasitic weed seeds showed variation in isolates ability to inhibit the germination of weed seeds. Eleven isolates were able to highly inhibit germination completely, whereas the rest of tested isolates allowed germination of up to 70%. In-vivo tests using semi-natural system of constructed plastic bags as well as pots (seeded with both faba bean and O. crenata) showed characteristic high mycoherbicidal activity of six Fusarium isolates. Two of which was identified as F. oxysporum, one as F. equiseti, one as F. verticillioides whilst the other two were identified as F. compactum. Two of which showed high pathogenicity and were significantly virulent to the bean broomrape. Such results suggest the potential of some Fusarium species which suppressed seed germination and parasitic establishment in bean broomrape to broaden the spectrum in the biocontrol of these problematic parasitic weeds.

Keywords - Faba bean, Crenate broomrape, Fusarium species, Mycoherbicides.

I. INTRODUCTION

Orobanche species are aggressive parasitic weeds that are highly distributed in East Africa, the Mediterranean basin, and the Middle East. Losses in yields infested by this weed may reach 100% based on several factors including host susceptibility, infestation level, and environmental conditions [1]. Control methods investigated have included changes in agricultural practices, as well as chemical and biological means. Biological methods to control Orobanche have been tested by various investigators on different crops but none of them was completed [2]. Bean broomrape (Orobanche crenata Forsk.) causes serious crop losses in legumes, especially faba bean (Vicia faba L.), lentils (Lens culinaris Medikus), forage legumes [3] and white lupine (Lupinus albus L.) [4]. Heavy infestation leads to field soils Orobanche-sick over a long period of time, affecting yield of legumes in such fields for many years [5]. It has been mentioned that faba bean being the most seriously affected food legume with O. crenata in North Africa, in Morocco and Algeria, Orobanche infestation in legumes reached 51 % and 60 %, respectively [6]. O. crenata alone accounts for more than 30% of the total losses caused by all other crop management constraints in Egypt [5]. Different biocontrol methods were applied to either destroy or reduce the parasite seed bank in infested soils with many researches focusing on Fusarium species which have been isolated in association with infected Orobanche species [7]. Interest in use of phytopathogenic fungi and/or their phytotoxic metabolites in biocontrol programs of weeds are growing up with a special concern on bioactivity of some Fusarium strains which was attributed to the variety of phytotoxic metabolites they produce and which may be used as natural heribicdes in broomrape management [8]. Many Fusarium species were evaluated against different Orobanche spp. and some has already been tested for interference with germination process of such parasitic weeds [9]-[11]. In addition, natural phytotoxic compounds produced by fungi were assayed for harbicidal activity in parasitic weed management strategies by targeting early growth stages of Orobanche. These metabolites could inhibit seed germination, so preventing attachment to the host plant or, conversely, stimulate seed germination in the absence of the host, contributing to a reduction in the parasite seed bank [12], [13]. Objectives of the present study were to survey, isolate biocontrol fungal agents from diseased Orobanche with a special focus on Fusarium species, to test and compare their phytotoxicity and pathogenicity against this weed using different bio-assays.

II. MATERIALS AND METHODS

Field survey and fungal isolates

Fungal isolates used in this study were obtained from shoots and inflorescences of young diseased O. crenata plants selected from masses infesting three main faba bean fields at Bani-Suef governorate (Upper Egypt). Fusarium isolates were identified using the taxonomic keys [14] - [17]. For microscopic

characteristic, isolates were cultured onto carnation leaf-pieces agar (CLA) for 2- 4 weeks [18]. Isolation of Fusarium from infected O. crenata plants Fusarium isolates were recovered from pieces (3-5-mm) cut from infected shoots and inflorescences of collected broomrape plants. These pieces were surface-sterilized with 1% sodium hypochlorite for 4 min., prior to plating on potato dextrose agar (PDA, Oxoid ltd. UK) amended with 250 µg mL^{-1} chloramphenicol (Sigma Chemical Co, St. Louis, MO, USA) and 100 µg mL^{-1} streptomycin sulphate (Sigma). Plates were incubated at 25°C±2°C in the dark for 8 days and were examined daily for fungal growth. Pure fungal cultures were maintained on PDA slants and stored at 4°C for use throughout the study.

Conditioning of Orobanche crenata seeds

Orobanche seeds were rinsed several times with sterile distilled water, dried overnight at room temperature, then placed in glass Petri dishes on wet GF/A (2.4 cm) Whatman glass microfiber filters (Whatman Int. Ltd., VWR, England). MES buffer drops (N-morpholino ethane sulfonic acid, GFS Chemicals Inc., Ohaio, USA) adjusted at pH 5.8 were used to wet the seeds before wrapping plates with aluminum foil and incubation in complete darkness for one month at 22° C. Filters were cut later into small pieces, each containing weighed Orobanche seeds (1 mg = 250-260 seeds).

Biological assay with fungal culture filtrates

Fusarium isolates were grown in still culture of potato dextrose broth (PDB) and incubated at 25±2°C in the dark for 5 days. Five mL from culture filtrate were passed through sterile millipore membranes (0.22 µm, Millipore Corporation, Billerica, MA, USA) to remove cell debris then two mL of each test filtrate were used to wet GF/A filters in plates with 2 mL of the synthetic germination stimulant (Nijmegen-1; 2 µg mL^{-1}) [19]. Filter papers loaded with hundreds of preconditioned broomrape seeds were placed over GF/A filters in each plate before sealing and incubating at 22±2°C for 7 days in the dark. Germination percentage of seeds was determined microscopically where seeds considered germinated only when their germ tubes were at least twice the seed length. Both germinated and non-germinated seeds were counted and expressed as a percentage in relation to the total number and compared to the control (seeds of O. crenata moistened with 2 mL sterile distilled water and Nijmigen-1).

Pathogenicity in semi-natural system " polyethylene bags"

Highly active Fusarium isolates were selected after seed germination test. According to [20], [21] Whatman GF/A glass fibre sheets (Whatman Int. Ltd., VWR, England) were placed in polyethylene bags (20 X 30 cm) wetted with 10 mL of Hoagland's solution. The solution was also used to fill glass tanks (1 cm depth) with sheets being fixed to both sides of the tank. O. crenata seeds were dispersed over the GF/A sheets in each bag in addition to three surface sterilized seeds of Vicia faba sprayed with culture filtrates of each test fungus. Polyethylene bags were placed upright with plant roots in the dark while their shoots emerge above the box, plastic covers were used to help plants retaining moisture. Tanks were placed for two weeks in a growth chamber set at 23±2°C with a 14 h day under fluorescent light (80 µmol $m^{-2}s^{-1}$) and observed every other day for germination signs, were replenished with 20 mL of 1/4 strength Hoagland's modified salt mixture (MP Biochemicals, Irvine, CA, USA) when needed. Bags were opened and the host roots with attached tubercles (6-8 mm) of O. crenata were sprayed with fungal inoculum (5×10^{8}CFU mL^{-1}) of each test isolate and incubated for another 3 days. Controls were set up with seeds of V. faba free of fungus.

Pathogenicity of selected Fusarium isolates to tubercles of O. crenata in pot trials

Three Fusarium isolates (BSHA7, BSHA9 and BSHF3) were used for pot trials following assay in polyethylene bags test. Faba bean and O. crenata seeds were surface-sterilized in 70% ethanol for 3 min., immersed in 1% sodium hypochlorite for 3 min. then washed several times with sterile distilled water. Beakers were filled with 500 g soil brought from infested fields to which 3 mg of O. crenata seeds were mixed with soil in each beaker before faba bean seeds (3 seeds/beaker) were sown [22]. Beakers were kept at 23°C±2°C with 14 h day (under 80 µmol $m^{-2}s^{-1}$ fluorescent light) and watered when needed. Three weeks later, the beakers contents were transferred to plastic pots (20 cm-diameter) filled with 4 kg of soil mixed with additional 7 mg of O. crenata seeds. Pots were supplied with a suspension (125 mL) of mycelium and conidia in sterile distilled water as fungal inoculum for each of the three selected isolates [23]. Pots were kept for one month in a greenhouse at 25°C±2°C, watered daily to container capacity and were fertilized weekly with Thrive® inorganic liquid fertilizer (Arthur Yates & Co Limited, Milperra, NSW, Australia) at the manufacturer's recommended rate. The experiment was conducted three times with three independent replicates per isolate, with control pots free from fungal inoculum. Underground tubercles formed on the host roots were dusted carefuly to remove soil and cleaned gently under tap water to record colour progress of infection in comparison to the control. To fulfil Koch's postulates, random pieces of diseased tissue of Orobanche shoots were surface-sterilized and the pathogen recovered as described above.

HPLC analyses of selected Fusarium culture filtrates

Ten mL from culture filtrates of selected Fusarium isolates (11 isolates) were exhaustively extracted with

ethyl acetate under acidic conditions (pH 2, HCOOH 1M). Organic extracts were combined, dried and then evaporated under reduced pressure before dissolving in methanol (5 mL) and passing through disposable filters where aliquots (20 µl) were prepared for chromatographic analyses. Spectroscopic detection was carried out by using HPLC (Shimadzu, Japan) with SPD-10 AD UV spectrophotometric detector and LCI 100 integrator on a reversed phase column RP-18 Licrospher 100, 5 µm particle size (250x4 mm). Elution condition used were that reported in [24], flow rate was 1 ml/min and detection wave length was at 268 nm where HPLC calibration curves for quantitative determinations were performed using solutions of FA standard (Sigma, St. Louis, MO, USA) in methanol in the range of "0.10 to 1.00" □g.
Statistical analyses

To evaluate the effect of the treatments on seed germination and on Orobanche tubercles, all data obtained were subjected to ANOVA and significant differences between means were determined using Fisher's Protected LSD Test at P = 0.05.

III. RESULTS

Field survey and fungal isolates
Infestation level with broomrapes in the three main faba bean surveyed fields (A, E and F) was moderately high with 3-5 O. crenata shoots surrounding the host stem (Fig. 1). Changes occur in the morphological features of faba bean plants infested with this weed could be detected in the infested fields as a reduction (%) observed in plant height, number of leaves, number of mature legumes. Total Fusarium isolates obtained from the infected parts in the young shoots and inflorescences of the collected broomrapes were fifty-one. Isolates of Fusarium were recovered and identified into ten species. Based on the morphological characteristics, the Fusarium species were identified as F. solani Mart. (20 isolates), F. oxysporum Schelcht (19 isolates), F. verticillioides (4 isolates), F. compactum sensu Gordon (2 isolates) and one isolate of: F. Sambucinum Fuckel, F. semitectum Berk. & Ravenel, F. subglutinans (Wollenw. & Reinking), F. proliferatum (Matsush.) Nirenberg, F. sporotrichioides Bilai and F. equiseti (Corda) Sacc. (Table 1).

Biological assay of Fusarium isolates
The highest seed germination % for O. crenata "78 %" was obtained with a concentration level of 2 µg mL^{-1} of the synthetic stimulant. Seeds were considered germinating when they gave germ tubes at least three times the normal seed length while non-germinated seeds were those having shorter ones or have not at all (Fig. 2). Activity of the 51 Fusarium isolates on seed germination of O. crenata was evaluated by counting non-germinated seeds then expressing them as percentage against the number of germinated ones in relation to the total seed number. Culture filtrates of eleven Fusarium isolates highly affected the germination of broomrape seeds with different inhibition ratios ranging from "68 to 92%" with a characteristic activity recorded for two F. compactum isolates and a F. oxysporum isolate. Sixteen isolates showed moderate or weak activity on seed germination where as the rest of isolates were considered inactive since they didn't inhibit the germination of even 25% of seeds. The interaction between the Orobanche species and the Fusarium isolates indicated that the Fusarium isolates differed significantly in the degree to which they inhibited seed germination of the two Orobanche species ($P<0.05$).

Pathogenicity of selected Fusarium isolates to O. crenata tubercles in polyethylene bags
Following incubation for ten days, treated V. faba seeds started to germinate normally in all plastic bags with no signs of germination for the O. crenata seeds recorded in treatments with six out of eleven tested isolates. The inhibition in broomrape seed germination was clearly observed in those plastic bags treated with culture filtrates of two F. compactum isolates (BSHA7 and BSHF3) and the F. oxysporum isolate (BSHA9). Broomrape tubercles developed from few germinating seeds on the host roots were turning brown or softly rot at the end of incubation time after spraying with fungal inocula (Fig. 3).

Pathogenicity of selected Fusarium isolates to tubercles of O. crenata in pot trials
The three tested Fusarium isolates were virulent but with different degrees to the tubercles of O. crenata developed on host roots. A characteristic aggressiveness was recorded for the F. compactum isolate (BSHF3) where the inoculum induced severe browning and softening in tubercles tissues (Fig. 4) by the end of the evaluation period and was recovered from all the diseased tissues examined.

HPLC analyses of selected Fusarium culture filtrates
Fusaric acid (FA) at a retention time of 11.09 min., was detected in only seven organic extracts out of eleven and with a retention time similar to that recorded for the authentic sample. Yield of FA ranged between "0.52 to 9.74" mg/100 mL, whereas recovery percentages for amounts of standard FA added to culture filtrates were between "94.6±3.1 to 101.3±2.8". The three F. oxysporum isolates (BINF9, BINE5, BSHA9) were the highest producers of FA with a production yield of 5.33, 6.28 and 9.74 mg of FA per 100 mL culture filtrate, respectively (Table 2).

IV. DISCUSSION
Broomrapes are among the most serious agricultural pests that attack strategic food crops, the continuous

spread of Orobanche limits the choice of rotational crops and often force farmers to give up growing the most profitable host crops [2]. The diversity of Fusarium species which has been isolated in association with diseased Orobanche species [24] increases attention towards biological control using these fungi which represent the largest component of the broomrape mycoflora [7], [11], [25], [26]. In this study, the major part of fungal isolates obtained from infected O. crenata parts was belonging to three main genera: Fusarium, Aspergillus and Alternaria. The total Fusarium count was around 2236 colony forming unit (CFU) representing 39 % of the total fungal CFU. Culture filtrates of eleven Fusarium isolates were found to inhibit the germination of crenate broomrape seeds in vitro by "68% to 92%". Six of these isolates hampered the development of this parasitic weed in semi-natural system using polyethylene bags in presence of host plants. Three out of these six were characteristically phytotoxic to broomrape seeds. Pathogenicity tests in pots containing highly infested soil cultivated with faba bean with such active isolates reflected their virulence. One isolate out of the three active ones namely F. compactum (BSHF3), induced significant rotting in broomrape tubercles developing on host plant roots and was not pathogenic to faba bean but moderatly improved its growth. This isolate was re-isolated from the infected tissues of Orobanche tubercles, meeting Koch's postulates for primary pathogens. Bioactivity of some Fusarium species was attributed to their ability to produce phytotoxins which may be considered as bio-herbicides in weed control [27]. Some authors recorded the ability of different strains of Fusarium for toxins (including trichothecenes) production, and for ability to inhibit O. ramosa seed germination. In particular, a F. compactum strain (ITEM 6160), isolated for the first time from O. ramosa widely spread throughout infesed fields in the Southeren of Italy, was found to produce a phytotoxic compound which was isolated and characterized as a trichothecene named neosolaniol monoacetae [28]. Fusaric acid was detected and quantified in seven organic extracts obtained from liquid culture filtrates of eleven selected Fusarium isolates which proved to highly phytotoxic to the germination of broomrape seeds. However, FA was absent in organic extracts of the two F. compactum culture filtrates. This may correlate their noticeable herbicidal activity especially that recorded for the isolate BSHF3 with strong pathogenicity to broomrape tubercles in pot trials, to the presence of other possible toxic metabolites recorded in a previous work [28]. Fusaric acid was reported as a secondary metabolite of Gibberella fujikuroi (Sawada) Wollenw. (current name Fusarium monoliforme), known for a long time to display strong phytotoxicity [29] and moderate toxicity to animal cells. However, more attention has to be directed to natural toxins as effecient tools in biological control programs but remain field trials under common agricultural practices to be first considered, prior to the application of a mycoherbicide in vivo. methods of formulation must be studied since the choice of formulation may increase or modify its effectiveness or host preference [30]. Formulation difficulty could be due to lack of literature on use of F. compactum as a mycoherbicide since most of the previous studies in this field concentrated only on F. oxysporum especially the strain F. oxysporum f. sp. orthoceras (FOO) [31] and Fusarium oxysporum F. sp. Orobanche [32] which has been promoted as a strong bioherbicidal candidate.

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Figures



Fig. 1. Collected Faba bean plant parasitized by four mature O. crenata shoots.

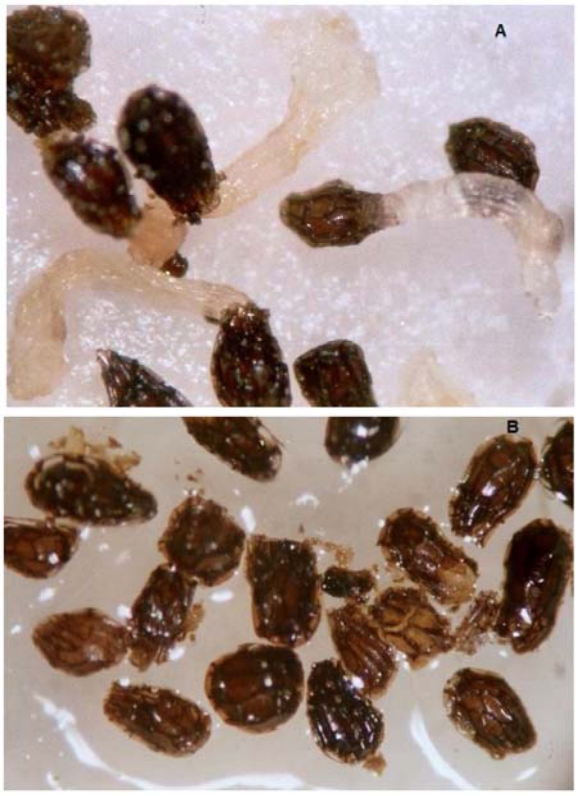


Fig. 2. Magnified microscopic images of crenate broomrape seeds showing difference between germinating (A) and non-germinating ones (B).

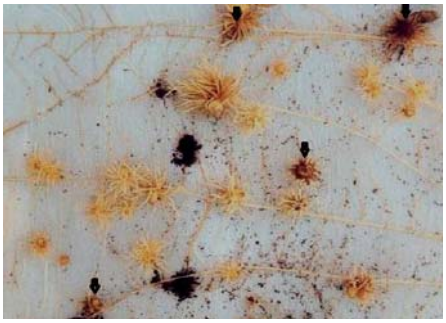


Fig. 3. Browning (indicated by black arrows) in Crenate broomrape tubercles developed on host roots in polyethylene bag system.

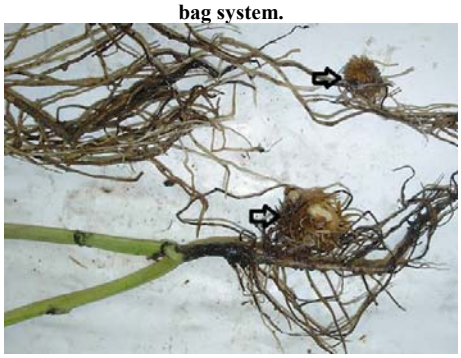


Fig. 4. Rotting and browning in tubercle's tissue of crenate broomrape developing in V. faba root in pot treated with the active isolate F. compactum (BSHF3) inoculum.

Table 1. Activity of isolated Fusarium species against Crenate broomrape: Phytotoxicity; Pathogenicity; and Assessment of fusaric acid's production.

Isolate	Species	OCSG	OCPP	OCPP	FAPA
BSHA1	***Fusarium solani***	**2**	**0**	**ND**	**-**
BSHA2	*Fusarium oxysporum*	0	-	ND	ND
BSHA3	*Fusarium oxysporum*	1	-	ND	ND
BSHA4	*Fusarium solani*	0	-	ND	ND
BSHA5	*Fusarium sambucinum*	1	-	ND	ND
BSHA6	*Fusarium solani*	0	-	ND	ND
BSHA7	***Fusarium compactum***	**3**	**2**	**2**	**-**
BSHA8	*Fusarium oxysporum*	0	-	ND	ND
BSHA9	***Fusarium oxysporum***	**3**	**3**	**2**	**+++**
BSHE1	*Fusarium oxysporum*	1	-	ND	ND
BSHE2	*Fusarium oxysporum*	0	-	ND	ND
BSHE3	*Fusarium solani*	1	-	ND	ND
BSHE4	*Fusarium solani*	0	-	ND	ND
BSHE5	*Fusarium verticillioides*	0	-	ND	ND
BSHE6	*Fusarium oxysporum*	0	-	ND	ND
BSHE7	*Fusarium sporotrichioides*	0	-	ND	ND
BSHE8	*Fusarium proliferatum*	1	-	ND	ND
BSHE9	***Fusarium equiseti***	**2**	**2**	**ND**	**-**
BSHE10	*Fusarium oxysporum*	0	-	ND	ND
BSHF1	*Fusarium verticillioides*	1	-	ND	ND
BSHF2	*Fusarium oxysporum*	1	-	0	ND
BSHF3	***Fusarium compactum***	**3**	**3**	**3**	**-**
BSHF4	*Fusarium solani*	0	-	ND	ND
BSHF5	*Fusarium oxysporum*	0	-	ND	ND
BSHF6	*Fusarium solani*	0	-	ND	ND
BSHF7	*Fusarium solani*	1	-	ND	ND
BSHF8	*Fusarium solani*	0	-	ND	ND
BSHF9	*Fusarium oxysporum*	1	-	ND	ND
BSHF10	*Fusarium solani*	0	-	ND	ND
BINA1	*Fusarium sambucinum*	0	-	ND	ND
BINA2	***Fusarium solani***	**2**	**0**	**ND**	**+**
BINA3	*Fusarium oxysporum*	1	-	ND	ND
BINA4	*Fusarium oxysporum*	1	-	ND	ND
BINA5	*Fusarium solani*	0	-	ND	ND
BINE1	*Fusarium solani*	0	-	ND	ND
BINE2	*Fusarium semitectum*	0	-	ND	ND
BINE3	*Fusarium solani*	1	-	ND	ND
BINE4	*Fusarium solani*	1	-	ND	ND
BINE5	***Fusarium oxysporum***	**3**	**2**	**1**	**++**
BINE6	*Fusarium verticillioides*	0	-	ND	ND
BINE7	*Fusarium solani*	0	-	ND	ND
BINE8	*Fusarium solani*	0	-	ND	ND
BINF1	***Fusarium verticillioides***	**3**	**2**	**1**	**++**
BINF2	***Fusarium solani***	**3**	**1**	**ND**	**+**
BINF3	*Fusarium solani*	0	-	ND	ND
BINF4	***Fusarium oxysporum***	**3**	**1**	**ND**	**+**
BINF5	*Fusarium subglutinans*	0	-	ND	ND
BINF6	*Fusarium solani*	1	-	ND	ND
BINF7	*Fusarium oxysporum*	0	-	ND	ND
BINF8	*Fusarium oxysporum*	1	-	ND	ND
BINF9	***Fusarium oxysporum***	**3**	**1**	**ND**	**++**

N.B. Isolate's code: B=Bani-Suef Governate; SH = Shoots; IN = Inflorescences; A,E,F = Codes for fields surveyed. OCSG = Orobanche crenata seed germination: 3 = 75-100 % of inhibition of seed germination; 2 = 50-74 %; 1 = 25-49 %; 0 = below 25% inhibition. OCPP = Orobanche crenata pathogenicity in polyethylene bags: 3 = excellent; 2 = good; 1 = fair; 0 = negative. OCPP = Orobanche crenata pathogenicity in pots: 3 = excellent; 2 = good; 1 = fair; 0 = negative. FAPA = Fusaric acid's Production analyses in liquid cultures: + = positive (produce FA); - = negative (not produce FA). Blue-colored rows indicate isolates scoring the highest activity on Crenate broomrape whereas yellow-colored cells refer to FA content in analyzed fungal culture filtrates.

Table 2. Content of fusaric acid (mg/100 ml) quantified by HPLC* in Fusarium culture filtrates and their % compared with the total organic extract.

Isolate Code	*Fusarium* Species	Total Extract (mg/100 mL)	FA (mg/100mL)	FA (%)	% FA Recoverd	
					LOD (5%)	LOD (100%)
BSHA1	*F. solani*	36.8	0.00	0.00 %	97.4±1.8	98.9±1.6
BSHA7	*F. compactum*	32.6	0.00	00.00 %	98.5.±1.4	99.4±1.2
BSHA9	*F. oxysporum*	44.2	9.74	22.03 %	97.1±2.1	100.4±2.9
BSHE9	*F. sporotrichioides*	43.5	0.00	00.00 %	96.7±2.1	99.7±1.5
BSHF3	*F. compactum*	29.8	0.00	0.00 %	98.3±1.1	101.3±2.8
BINA2	*F. solani*	21.4	0.52	0.02 %	94.6±3.1	96.6±2.6
BINE5	*F. oxysporum*	37.6	6.28	16.70 %	99.3±3.1	100.1±2.4
BINF1	*F. veticillioides*	28.2	3.41	12.09 %	97.4±1.6	98.8±2.3
BINF2	*F. solani*	57.8	5.16	8.92 %	98.1±2.6	99.4±1.1
BINF4	*F. oxysporum*	38.6	3.11	8.05 %	95.7±1.5	96.1±1.2
BINF9	*F. oxysporum*	43.8	5.33	12.16 %	98.8±2.1	99.6±2.1

*Chromatographic run consisted of methanol (A), water (B) and 1% dipotassium hydrogen phosphate in water (pH 7.25) (C). Run started with A:B:C (55:10:35) which was changed through a linear gradient over 20 min. to A:B:C (70:10:20); with restoring the initial conditions over 5 min.; LOD: Limit of detection calculated as 3×S.D.Y/S (S: chromatographic peak area/ µg of metabolites for the lower amount of the linear range).

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