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Screening the antifungal activity of soilborne actinomycetes

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Abstract

A total of 288 actinomycete strains, isolated from 35 soil samples collected from western Turkey, were tested against pre- and post-harvest fungal pathogens such as *Aspergillus niger* (ATCC-1094), *A. parasiticus* (NRRL-465), *Fusarium moniliforme* (NRRL 1866), *F. solani* (NRRL-13414), *Penicillium chrysogenum* (NRRL 807), *A. fumigatus*, *Alternaria citri*, *Al. tenuissima*, *Athelia rolfsii*, *F. acuminatum*, *F. aveneceum*, *F. clamydosporium*, *F. culmarum*, *F. equiseti*, *F. oxysporum*, *F. semitectum*, *F. subglutanas*, *Fusarium* sp., *Gaeumannomyces graminis*, *Mycosphaerella rabiei*, *P. citrinum* and *P. piceum*. During primary screening, 30.55% of the strains showed inhibition potential against the test pathogen fungi. Of the effective isolates, seven strains were selected for secondary screening with four different fermentation media based on their high inhibition potential in primary screening. The cell-free supernatant of fermentation B medium showed broad-spectrum and high inhibition potential against the test fungi. After extraction with ethyl acetate, XF, BF, AM3.1, 9M and 14M from the selected seven strains exhibited antifungal activity against particularly some members of *Fusarium* and *Aspergillus* genera. These results indicate that the tested five actinomycete strains have potential activity against pre- and post-harvest pathogens, and their antagonist compounds can be produced and obtained without the loss of antifungal effects, which could be useful for the protection of agricultural products.

Key words: actinomycete, antifungal activity, plant pathogens, soil, screening

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Toprak kaynaklı aktinomisetlerin antifungal aktivitesinin taranması

Özet

Türkiye'nin batısından toplanan 35 toprak örneğinden izole edilen toplam 288 aktinomiset suşu *Aspergillus niger* (ATCC-1094), *A. parasiticus* (NRRL-465), *Fusarium moniliforme* (NRRL 1866), *F. solani* (NRRL-13414), *Penicillium chrysogenum* (NRRL 807), *A. fumigatus*, *Alternaria citri*, *Al. tenuissima*, *Athelia rolfsii*, *F. acuminatum*, *F. aveneceum*, *F. clamydosporium*, *F. culmarum*, *F. equiseti*, *F. oxysporum*, *F. semitectum*, *F. subglutanas*, *Fusarium* sp., *Gaeumannomyces graminis*, *Mycosphaerella rabiei*, *P. citrinum* ve *P. piceum* gibi hasat öncesi ve sonrası fungal patojenlere karşı test edilmiştir. Birinci taramada, test edilen suşların % 30,55'i fungal patojenlere karşı inhibisyon potansiyeli göstermiştir. Etkin izolatlardan 7 suş birinci taramada yüksek inhibisyon potansiyellerine dayalı olarak dört farklı fermantasyon ortamı ile ikinci tarama için seçilmiştir. Fermantasyon B ortamının hücresiz süpernatantı test funguslarına karşı geniş spectrum ve yüksek inhibisyon potansiyeli göstermiştir. Etil asetat ile ektraksiyondan sonra, seçilen yedi suştan XF, BF, AM3.1, 9M ve 14M özellikle bazı *Aspergillus* ve *Fusarium* üyelerine karşı antifungal aktivite göstermişlerdir. Bu sonuçlar; test edilen beş aktinomiset suşunun hasat öncesi ve sonrası fungal patojenlere karşı potansiyel aktiviteye sahip olduğunu, zirai ürünlerin korunması için kullanışlı olabilecek antagonistik bileşiklerinin üretilebilir ve antifungal aktivitelerini kaybetmeden elde edilebilir olduğunu işaret etmektedir.

Anahtar kelimeler: aktinomiset, antifungal aktivite, bitki patojenleri, toprak, tarama

1. Introduction

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Fungi are a commonly diverse group with a wide range of habitats and have several functions, ranging from the decomposition of organic materials to pre- and post-harvest pathogenesis of food products as well as the production of mycotoxins (Demirel et al., 2013; Asan, 2015). In particular, fungal phytopathogens have a worldwide distribution with large economic and health effects on human, animal and plant life (Oksay, 2009; Samson et al., 2014; Visagie et al., 2014). Conventional approaches such as chemical fungicides have been used for the control of fungi; however, these chemicals have toxic effects for living organisms and lead to environmental pollution (Oksay, 2009; Şengün and Yücel, 2015). Hence, natural compounds are indicated for reducing the development of pathogenic fungi and for controlling pollution (Kumar et al., 2012; Sen and Batra, 2012). Actinomycetes are one of the most attractive sources of several types of bioactive metabolites such as antibiotics (Nurkanto et al., 2012; Saxena et al., 2013; Sharma et al., 2014; Kaur et al., 2015). Their antifungal antibiotics have a significant role in the control of fungal pathogens. Therefore, actinomycete-based biocontrol approaches are one alternative way to control plant infections of agrochemicals (Oksay, 2009; Saxena et al., 2013; Kaur et al. 2015).

Only a limited number of antifungal agents are presently available against fungal pathogens (Sharma et al., 2014). The search for new, safe and broad-spectrum antifungal agents has become important not only for agricultural protection but also for human health. Accordingly, the objectives of the present study were the isolation of actinomycetes from soil habitats, screening their antifungal activity against pre- and post-harvest fungal pathogens and assessing the possibility of the extraction of antifungal metabolites from potential actinomycetes.

2. Materials and methods

1.1. *Test microorganisms*

Some standard test fungi such as *Aspergillus niger* (ATCC-1094), *A. parasiticus* (NRRL-465), *Fusarium moniliforme* (NRRL 1866), *F. solani* (NRRL-13414) and *Penicillium chrysogenum* (NRRL 807) and wild-type test fungi such as *A. fumigatus*, *Alternaria citri*, *Al. tenuissima*, *Athelia rolfsii*, *F. acuminatum*, *F. aveneceum*, *F. clamydosporium*, *F. culmarum*, *F. equiseti*, *F. oxysporum*, *F. semitectum*, *F. subglutanas*, *Fusarium sp.*, *Gaeumannomyces graminis*, *Mycosphaerella rabiei*, *P. citrinum* and *P. piceum* were used as test microorganisms. The common feature of the tested microorganisms is that they are all pre- and post-harvest pathogen fungi.

1.2. *Collection of soil samples*

A total of 35 soil samples from Central Anatolia [Ankara (7 samples), Eskişehir (10 samples; 2 of them from boron fabric area)], North Anatolia [Bolu (3 samples), Düzce (6 samples), Samsun (1 sample)], Northwest Anatolia [Balıkesir (2 samples)], South Anatolia [Antalya (1 sample), Burdur (1 sample), Isparta (1 sample)], West Anatolia [Afyon (1 sample), Muğla (2 samples)] and the Turkish Republic of Northern Cyprus (TRNC) (1 sample) were collected according to Kitouni et al. (2005). Samples were stored at 4°C until analysis.

1.3. *Isolation of actinomycetes from soil*

The soil samples were mixed with 0.1 g $CaCO_3$ and air-dried at 28°C for 1 week. Then, about 10 g of soil sample was transferred to 90 ml physiological saline solution (NaCl 9 g l^{-1}). The soil suspension was further diluted to 10^{-5} dilution level. For inoculation of soil samples, pour plate technique (Murray et al., 1995) was implemented on nutrient agar (NA) and glycerol yeast agar (GYA) media. After incubation at 28°C for 5 days, the colonies of actinomycetes were recognised according to their macroscopic characteristic and then picked out and purified by repeated streaking on NA or GYA according to their growing medium. The pure cultures of actinomycetes were preserved at 4°C.

1.4. *Screening of antifungal activity*

Primary screening: Preliminary screening for antifungal activity was performed with conventional spot inoculation method (Yamamura et al., 2003), with some modifications. Pure actinomycete strains were spot inoculated with about 1 cm diameter in the centre of yeast malt extract agar (YMA) medium. After incubation at 28°C for 5 days, 6-mm plugs of 5-day-old cultures of test fungi were cross plugged 30 mm away on either side to the original plug of actinomycete isolates. The control plates were also maintained with each of the test fungi. All the plates were incubated at 28°C for 5 days and then the zones of inhibition of fungal growth were measured. From preliminary screening, strains of high antifungal activity were selected for secondary screening.

Secondary screening: The selected actinomycete isolates were further tested in secondary screening under submerged fermentation conditions by agar well method (Murray et al., 1995). Selected isolates were grown on four different liquid media, yeast-malt broth (YMB) (yeast extract 4 g l^{-1}, malt extract 10 g l^{-1}, glucose 4 g l^{-1}, pH 7.3), fermentation medium A (NaCl 0.8 g l^{-1}, NH_4Cl 1 g l^{-1}, KCl 0.1 g l^{-1}, KH_2PO_4 0.1 g l^{-1}, $MgSO_4.7H_2O$ 0.2 g l^{-1}, $CaCl_2.2H_2O$ 0.04 g l^{-1}, glucose 2 g l^{-1}, yeast extract 3 g l^{-1}, pH 7.3), fermentation medium B (glucose 10 g l^{-1}, bacteriological peptone 5 g l^{-1}, malt extract 5 g l^{-1}, NaCl 5 g l^{-1}, pH 6.5–7.0) and fermentation medium C (soya flour

extract 5 g l^{-1} (obtained by boiling 20 g soya flour in 100 ml distilled water for 1 h and filtered using a cheese strainer), glucose 5 g l^{-1}, bacteriological peptone 2.5 g l^{-1}, NaCl 2.5 g l^{-1} in tap water, pH 7.0).

Initially, pure actinomycete isolates were inoculated in 10 ml fermentation medium to obtain vaccine culture. This vaccine culture was incubated at 28°C under shaking at 200 rpm for 48 h. After incubation, 2.5 ml of prepared vaccine culture was transferred to 50 ml of the same fermentation medium and incubated at 28°C under shaking at 200 rpm for 48 h. To obtain the cell-free supernatant, the culture broth was centrifuged at 4000 rpm for 15 min at 4°C.

For screening of antifungal activity of the cell-free supernatant, each of the test fungi was inoculated into 50 ml YMA medium, with addition of 1 ml of 10^6 spore suspension, mixed well and then poured into sterile Petri dishes. About 5 ml of the inoculated YMA medium was kept in an Erlenmeyer flask for control of tested fungi. After drying the inoculated YMA plates, the agar wells (6 mm diameter) were cut using a sterile cork borer and then 50 µl of clear fermented broth was loaded into each well. The diameter of the zone of inhibition for each fungus was recorded after incubation at 28°C for 72 h. The strains and media of high antifungal activity were selected.

1.5. *Extraction of antifungal metabolites*

The selected isolates and fermentation media were used for extraction of antifungal metabolites. To this end, 1 L culture broth was prepared as above. The total cell-free supernatant, with pH adjusted to 4.0, was used for solvent extraction with ethyl acetate. A volume of cell-free supernatant and solvent (1:1 v/v) was taken in a separating funnel and shaken vigorously. Extraction was continued for up to three times. Subsequently, the organic layer was collected and the solvent was evaporated using a vacuum rotary evaporator at 40°C (Duraipandiyan et al., 2010).

1.6. *Bioautographic method using thin-layer chromatography*

The ethyl acetate extract was spotted onto thin-layer chromatography (TLC) plates coated with silica gel (60 F_{254}, Fluka). The chromatogram was developed in methanol. The plates were run in duplicate; one of them was used as a reference plate and the other plate was used for bioautography. After development, the fluorescent compounds were observed at 365 nm in an ultraviolet chamber. For bioautography, the TLC plate was placed on YMA Petri dish and 5 ml top agar was poured, including the test fungi with 200 µL of 10^6 spore suspensions. After incubation at 28°C for 5 days, the zones of inhibition were investigated by using the reference plate (Selvameenal et al., 2009).

3. Results

Plant and plant product diseases caused by fungi are one of the primary concerns of agricultural production. The use of microorganisms or some of their metabolites for protection of plants and their products is a common biological control method against fungal pathogens. Therefore, discovering bioactive agents from different habitats has become an important approach (Kathiresan et al., 2005; Oskay, 2009; Nurkanto and Julistiono, 2014). Actinomycetes are the primary source for antifungal agents and are also well known as biocontrol agents, such as members of *Streptomyces* genus. The major habitat for obtaining bioactive actinomycetes is soil (Nurkanto et al., 2012; Sharma et al., 2014). Therefore, in the present study, we focused on soilborne actinomycetes and their antifungal activities.

A total of 288 strains were obtained from six different regions of Turkey. The maximum number of isolates and active isolates obtained achieved from Central Anatolia region, followed by North Anatolia, Northwest Anatolia, West Anatolia and South Anatolia, corresponding to the number of investigated soil samples. From the TRNC, we could not obtain actinomycetes (Figure 1).

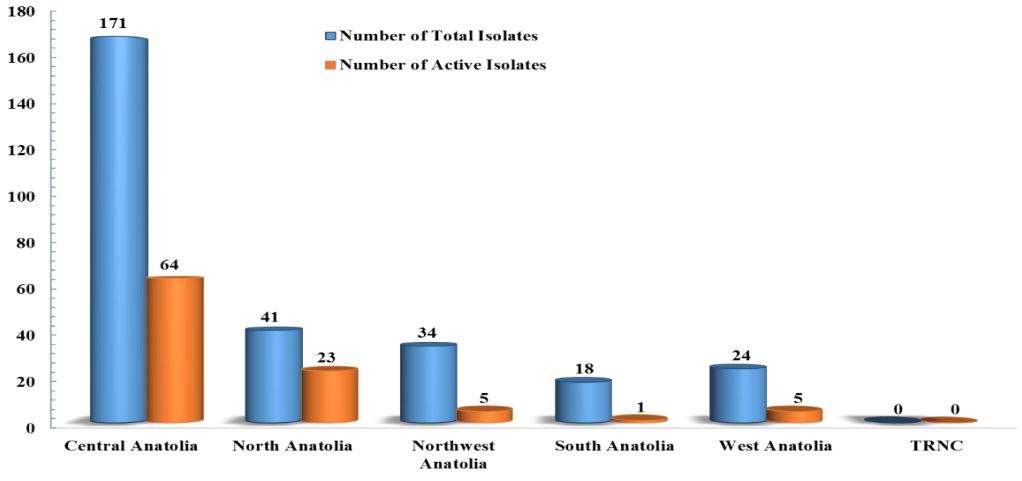

Figure 1. The number of total and active isolates according to regions

The results of primary antifungal screening assay showed that 88 of the 288 tested actinomycetes (30.55%) had some antagonist activity against filamentous fungi, including pre- and post-harvest product pathogens (Figure 1). Of the 88 active isolates, about 12% were effective against *F. solani* (NRRL-13414) and about 11% were antagonist against *F. culmorum*. Only 10% of active strains were effective against *F. moniliforme* (NRRL 1866) (10%), followed by other tested filamentous fungi (0–8%) (Figures 2 and 3). Of the effective isolates, only seven strains, five (BF, BB, 9M, 14M, 34M) from Central Anatolia (Eskişehir; boron soil), one (XY) from North Anatolia (Samsun) and the other one (AM3.1) from Northwest Anatolia (Balıkesir), were selected for secondary screening based on their high inhibition potential. Several studies have been conducted regarding the screening of antimicrobial activity of actinomycetes isolated from different types of soil samples and sediments. One of the interesting results of these studies is that industrially polluted sites showed the highest incidence of antagonist actinomycetes (Walker and Colwell, 1975; Kathiresan et al., 2005). Based on the primary screening, numerous antifungal actinomycetes were isolated from Eskişehir boron fabric area at Central Anatolia. On the other hand, boron is toxic for living cells when present above a certain level. Hence, only boron-tolerant microorganisms can survive in this kind of natural environment, and such microorganisms comprise potential genetic sources for boron resistance and protection of agricultural products in naturally boron-contaminated area (Yoon et al., 2010).

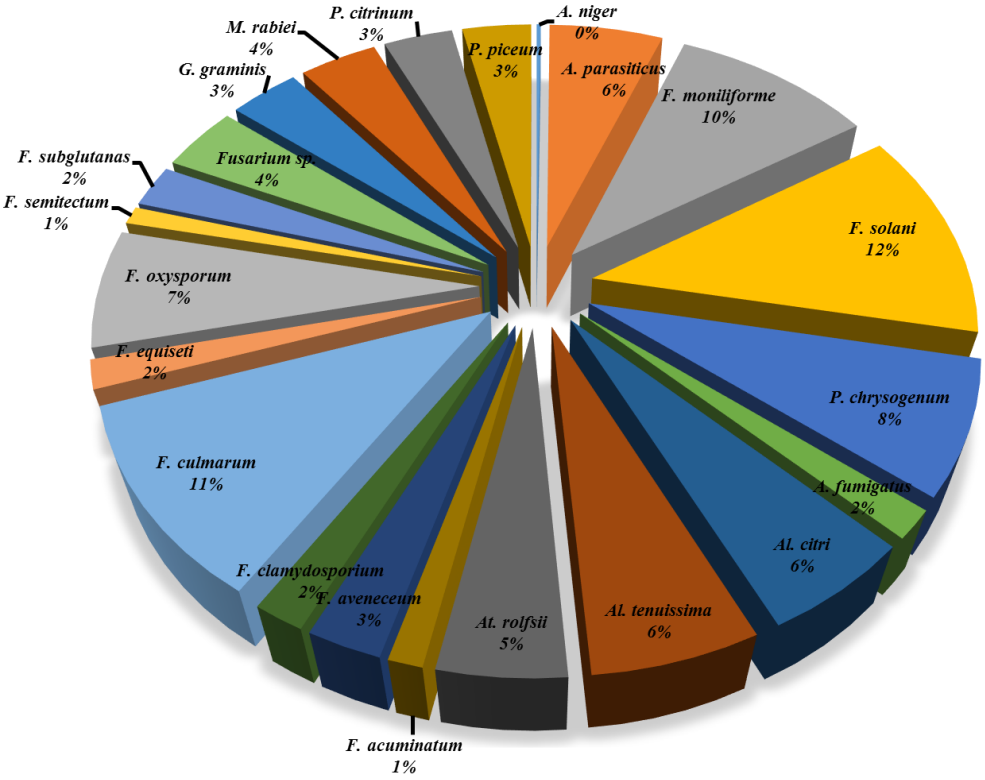

Figure 2. Primary screening results of actinomycetes isolated from soil samples

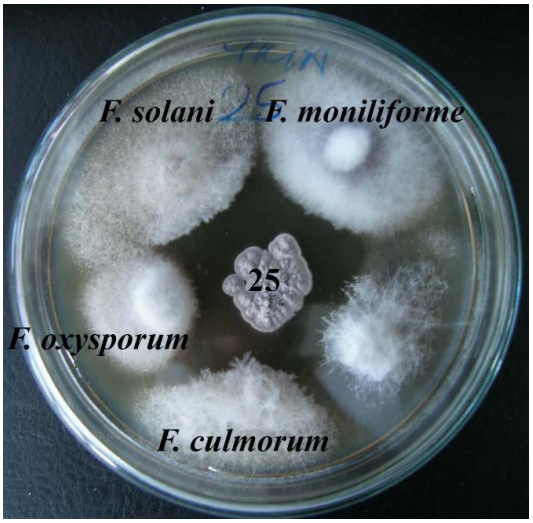

Figure 3. Antifungal activity of one of the actinomycete on tested pathogen fungi

The antifungal activity of the seven strains from the liquid culture of four different media was tested against *Al. citri*, *Al. tenuissima*, *A. niger* (ATCC-1094), *A. parasiticus*, *At. rolfsii*, *F. culmorum*, *F. moliniforme* (NRRL 1866), *F. solani* (NRRL-13414), *G. graminis*, *P. chrysogenum*, *P. piceum* and *M. rabiei*. The obtained broad-spectrum and high inhibition potentials were recorded on fermentation B medium that had the highest glucose content (Table 1) (Figure 4). However, the strains were less active or non-active on YMB fermentation A and C media that contained low glucose content than fermentation B medium and some extracts, inorganic compounds, soya flour extract, respectively. Kathiresan et al. (2005) have assessed the effect of carbon and nitrogen source on antifungal activity and reported that glucose was the best source.

Table 1. Antifungal activity of selected actinomycetes in the secondary screening (Fermentation medium B) (mm)

Isolates	***Al. citri***	***Al. tenuissima***	***A. niger***	***A. parasiticus***	***At. rolfsii***	***F. culmorum***
BF	0	0	0	0	0	0
AM3.1	0	0	0	0	0	0
34M	0	7	0	0	3	6
14M	0	0	0	0	0	3
BB	0	0	0	0	0	1
9M	2	1	3	2	4	4
XY	4	6	2	2	6	4
Isolates	***F. moliniforme***	***F. solani***	***G. graminis***	***P. chrysogenum***	***P. piceum***	***M. rabiei***
BF	0	5	0	0	0	1
AM3.1	0	1	0	0	0	0
34M	5	10	2	5	1	3
14M	0	6	0	0	0	2
BB	0	4	2	0	2	2
9M	3	6	4	2	2	1
XY	4	8	4	5	4	3

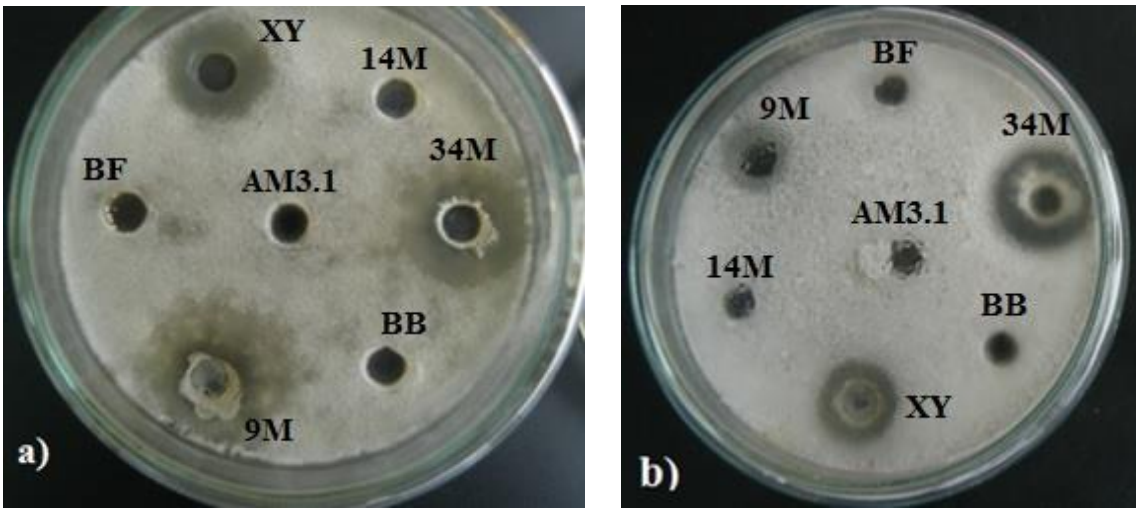

Figure 4. Antifungal activity against *Penicillium chrysogenum* (a) and *Fusarium moliniforme* (b) of cell free supernatant of fermentation B medium

The antifungal metabolites from the cell-free supernatant of fermentation B medium were extracted by using ethyl acetate, which is one of the favoured organic solvents (Duraipandiyan et al., 2010), and tested against *F. culmorum*, *F. moliniforme* (NRRL 1866), *F. solani* (NRRL-13414) and *A. parasiticus* by using bioautographic method. For this purpose, antifungal activity of crude extracts was determined, which showed that XF, BF, AM3.1, 9M and 14M from selected strains had antifungal activity against the tested filamentous fungi (Figure 5). In particular, crude extract of AM3.1 strain achieved broad-spectrum inhibition potential. The crude extracts of XF and BF strains were effective against *F. culmorum*, *F. moliniforme* and *F. solani*. The crude extracts of 9M and 14M exhibited antifungal activity against only *A. parasiticus*.

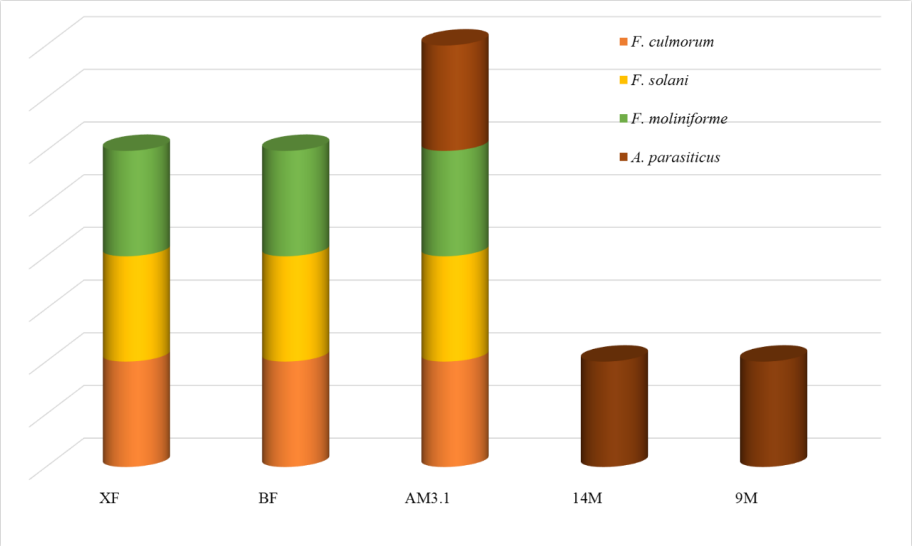

Figure 5. Antifungal activity of crude extracts against tested filamentous fungi

One-third of the total losses because of plant diseases is due to fungal infections (Oskay, 2009). The top 10 plant pathogen fungi have been listed as (1) *Magnaporthe oryzae*; (2) *Botrytis cinerea*; (3) *Puccinia* spp.; (4) *Fusarium graminearum*; (5) *Fusarium oxysporum*; (6) *Blumeria graminis*; (7) *Mycosphaerella graminicola*; (8) *Colletotrichum* spp.; (9) *Ustilago maydis* and (10) *Melampsora lini* by Dean et al. (2012). In addition, some members of *Penicillium*, *Aspergillus*, *Alternaria*, *Athelia* and *Gaeumannomyces* genera are very important for both pre-harvest and post-harvest infections and mycotoxin production (Samson et al., 2014; Visagie et al., 2014). Due to the increasing world population, it is necessary to control fungal diseases that reduce the agricultural crop yield. There are some conventional approaches such as chemical fungicides, but they have damaging effects on humans and other non-target organisms due to their toxicity (Oksay, 2009). Therefore, discovery of natural bioactive organisms and metabolites has become more important for use in non-offensive agrochemicals. This study showed that the tested five actinomycetes have a potential activity against pre- and post-harvest fungal pathogens, and their antagonist compounds can be produced and obtained without the loss of antifungal effects, which could be useful for several applications such as the protection of plant and plant products and discovery of new preparations.

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