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IN VITRO ANTAGONISM BETWEEN SOILRHIZOSPHERE MICROORGANISMS AND *A. Strictum* THE CAUSAL FUNGUS OF ACREMONIUM WILT IN GRAIN SORGHUM.

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ABSTRACT

Population densities of bacteria, sporeformers, actinomycetes and fungi were enumerated in soil and rhizosphere samples collected from fields cultivated either with a susceptible(Giza-113) or a resistant (Dorado) grain sorghum cultivars after 15,30,45, and 90 days of sward establishment. Results revealed that the rhizosphere of both cultivars harboured higher counts of the examined microorganisms than those found in soil apart .Counts of total bacteria, spore-formers and fungi increased as the plant growth increased till the45-day period and gradually decreased thereafter. Bacteria were the highest followed by actinomycetes, fungi and sporeformers being 34.2, 8.1, 0.6 and 0.3 x 10^6 cfu/g soil,respectively. During the earlier plant growth periods, counts of each type of microorganisms increased in the rhizosphere of both cultivars being higher with resistant plants.Out of 367 isolates (291 bacteria, 51 actinomycetes and 25 fungi) isolated from soil and the rhizosphere of Dorado and Giza 113 plants, 183 isolates (151 bacteria and 32 actinomycetes) proved to be effective against A. strictum. Twenty isolates of bacteria (identified *as Bacillus spp.*) and 9 isolates of actinomycetes (identified as *Streptomyces spp.*) showed the highest antagonistic effect. Isolate No. (9) of *Bacillus spp.* (identified as *B. subtilis*) and *Streptomyces spp.* No. (3) were the most antagonistic isolates. Culture filtrates of both bioagents individually singly or in combination (1:1) inhibited the mycelial growth of *A. strictum.*

Keywords: *Grain sorghum, Rhizosphere ,soil microorgisms, Antagonism, A.strictum .*

INTRODUCTION

Grain sorghum occupies a unique position among all other cereal rops in Egypt and all over the world. The crop is subjected to various diseases that cause a considerable loss in yield and affect the quality of grains, the most important of which is Acremonium wilt caused by the soil-borne fungus, *Acremonium strictum.* Biological control of such disease may be achieved through a number of strategies. The direct approach involves the isolation and incorporation of specific microbial antagonists to soil or planting materials (Yates *et al.*, 1999).

Some soil microorganisms have been reported to produce antagonistic substances that inhibit the pathogen(s) within the root environment of tomato (Ibrahim *et al.*,1996) , strawberry (Marten *et al.*, 2001) , cotton (Hassan,2001),.and grain sorghum (Ibrahim and Zein El-Abedeen,2000). However, a possible relationship has been suggested by many investigators between population densities and the antagonistic potentials of

rhizosphere microorganisms as well as plant genotype. In this regard, Asran (2001) found that populations of bacteria and actinomycetes in rhizosphere of healthy cotton cvs. seedlings were higher than that of diseased plants. The opposite was true when fungal population was estimated. Moreover, Habib (1979) stated that the antagonistic potentials of some microbial isolates were higher in the rhizosphere of less susceptible lentil cultivar than the more susceptible one.

Screening for a potent microorganism with potential pathogenic capabilities against causal pathogen(s) is a crucial step towards development of a successful biocontrol agent. A number of authors have stressed the importance of appropriate screening procedures (Weller, 1988; Jensen *et al.*, 1996 ; Knudsen *et al.*, 1997). Mixtures of biocontrol microorganisms in the form of different genera or even different species within the same genus were used to achieve synergism and persistent control as has been proven by Deacon (1994).

In the present study efforts were directed towards search and selection for a possible potent microbial candidate(s) against the pathogenic fungus *A. strictum* and finally identified to the genus or species level.

MATERIALS AND METHODS

Enumeration and isolation of microorganisms from soil and rhizosphere of susceptible and resistant grain sorghum plants

Population densities were enumerated using pour plate method of Parkinson *et. al.* (1971) for counting total bactria and sporeformers using soil extract yeast medium (Mahmoud,1955),actinomycetes on glycerol casein agar medium(Küster and Williams, 1964) and fungi on Martin's agar medium (Johanson *et al.*, 1959). Soil and rhizosphere samples of the resistant (Dorado) as well as susceptible (Giza-113) grain sorghum cultivars grown in the field at Giza experimental station, Agric .Res. Center during 2001's season were collected after 15, 30, 45, 60, 75 and 90 days of sowing and microbiologically analyzed.

Plants were gently uprooted and vigorously shaken to get rid of excess soil. The rootlets with the remaining adhered soil particles were added to 90 ml sterile distilled water in 250 ml-conical flasks. Flasks were shaken on a reciprocal shaker for 15 min. and serial dilutions were made from which three replicate plates were inoculated. Dilutions used for counting spore-formers were pasteurized at 80 °C for 15 min.

Colonies developed on plates incubated at 30°C were counted after 4 days for bacteria and sporeformers, 7 days for fungi and 10 days for actinomycetes. Well separated colonies were picked and examined microscopically for purity.Then, maintained under refrigeration for further studies on nutrient agar for bacteria and actinomycetes and on potato dextrose agar(PDA) for fungi.

Screening of microbial isolates for antibiosis twards *A. strictum*

The isolated miroorganisms (291 bacteria, 51 actinomycetes and 25 fungi), were tested for antagonism against *A. strictum* according to

Brock(1973) on PDA medium containing 0.5% peptone. Plates were incubated at 28 ± 2 °C and the inhibition zone diameters were measured after 5 days.The most antagonistic isolates were selected and maintained on their respective media.

Identification of the most effective isolates against *A. strictum* was carried out according to Buchanan and Gibbons (1974) for bacteria and Barnett (1960) and Buchanan and Gibbons (1974) for the actinomycets.

Effect of culture filtrates of effective isolates against *A. strictum*

One hundred ml broth cultures of the most antagonistic isolates, *Bacillus subtilis* (isolate No. 9) and *Streptomyces* sp. (isolate No. 3) were prepared in nutreint broth and starch nitrate broth (Waksman,1961), respectively, in 250 ml conical flasks and incubated on a rotary shaker (180 rpm) at 25 °C. Two-day old bacterial culture and 10-day old actinomycetal one were centrifuged (3000 rpm) for 10 min. and supernatens were filter-sterilized using sterile, non-pyrogenic and hydrophilic cellulose acetate syringe filter of 25 mm in diameter and 0.2 µm pore size [ALBET-JACS-020-25, Spain].

Non-diluted culture filtrates and broth diluted (1:1 and 1:3) preparations of each culture filtrate in addition to a 1:1 mixture of the two non-diluted culture filtrates were prepared and tested against *A. strictum*. Two different procedures of agar diffusion method (Brock, 1973) were applied. In the first, four sterilized filter paper discs (6 mm dia.), each was saturated with 0.2 ml of sterilized non-diluted culture filtrate of each treatment, were placed at equidistance on a Petri dish containing *A. strictum* inoculated medium. In the second, the above-mentioned four culture filtrate preparations were examined. Four cylinder wells (6.0 mm dia.) were made in the *A. strictum* inoculated medium, surrounding the center of the Petri dish using sterile cork borer. Then, 0.2 ml of each culture filtrate preparations was transferred separately into a well using sterile micropipett. Three replicate plates from each treatment were prepared using both methods and incubated for 5 days at 28 ± 2 °C.

RESULTS AND DISCUSSION

Enumeration and isolation of microorganisms in soil and rhizosphere of susceptible and resistant sorghum plants

Figure (1) shows that populations of bacteria, spore-formers and fungi increased as the plant growth increased till the 45th day in the rhizosphere of both cultivars, thereafter, they gradually decreased. Counts, in general, were much higher in the rhizosphere in soil apart. The dense microorganisms in the rhizosphere might be attributed to root exudates and high moisture content.

Total bacteria recorded the highest value among other microorganisms at the 45th day period, followed by actinomycetes, fungi and spore-formers being 34.2, 8.1, 0.6 and 0.3 x 10^6 cfu/g soil. Numbers were gradually decreased till the end of the experimental interval. Similar findings were reported regarding type and densities of microorganisms in the rhizosphere of tomato (Mohamed, 1997) and maize (Ebrahim, 2002).

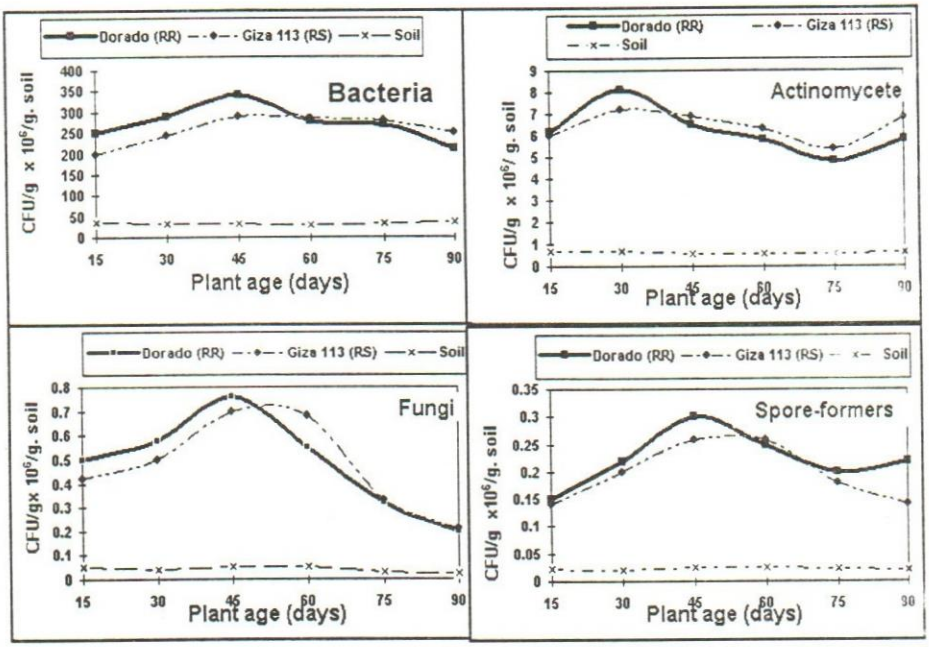

Figure (1) :Counts of microorganisms (cfu.g^{-1}) in the rhizosphere and soil of a resistant and a susceptible sorghum cultivars at different growth stages.

As to spore-forming bacteria, much lower numbers were recorded compared to the other groups in the rhizosphere of resistant and susceptible plants and in soil apart as well.This was not surprising as the soil under investigation is heavy textured and fertile.

With respect to actinomycetes, a different trend was observed. Their numbers increased in the rhizosphere of both cultivars as plants aged from 15 to 30 days, then decreased up to 75 days of plantation. An obvious increase in their population was again recorded at the late stage of plant growth. This is in accordance with Alexander (1982) and McCaarthy and William (1990) reports that actinomycetes persist during microbial succession beyond initial phase of population growth due to excretion of a wide range of hydrolytic exoenzymes. The ability of actinmycetes to penetrate root debris and similar tissues that might be present in plant rhizosphere and consequently solubilized cellulosic, hemicellulosic and humic materials was well demonstrated (Ebrahim, 2002). Certainly, such stimulating effect could be considered, as an additional evidence, to explain the active growth and the high viable cell counts recorded for all types of microorganisms over the first 30 - 45 days of plantation.

On the other hand, plant age was found to have a very pronounced influence on growth performance of all rhizospheric microorganisms. Over the whole cultivation period, such influence displayed a typical patterns, characterized by a biphasic nature, with exponential growth phase till the 30th day for actinomycetes and the 45th day for the others, followed by a decline

phase for growth towards the end of cultivation. During the first phase, viable cell counts of each type of microorganisms increased in the rhizosphere of both cultivars but with resistant plants being higher. Such differences between cultivars might be due to ability of the resistant cultivar to excrete specific root exudates that possibly stimulate growth of microorganisms in rhizosphere (Cook *et.al.*, 1996).

During the decline phase, results showed a reverse trend for all microorganisms, except spore-formers. Counts were higher in the rhizosphere of susceptible plants compared to resistant ones. This is most probably due to a possible deformation of the susceptible plant roots as a result of infection with *A. strictum*. Being a predisposing agent (El-Shafey *et al.*, 1999), *A. strictum*-infected roots could easily be invaded by other microorganisms including fungi and spore-formers. It is well known that spore-formers and certain soil fungi play together a significant role in the disintegration of internal tissues of diseased plants (Sabet *et al.*, 1966). This might have resulted in the release of some nutrients and sloughing of organic materials that made the environment around the diseased roots more suitable for growth of the majority of microorganisms in comparison to those surrounding healthy roots. These results are in conformity with those obtained by El-Shafey *et al.* (1985). Unlike the other microorganisms, both fungi and spore-formers behaved differently during this period. The fungal counts were markedly reduced, reaching lower values being 40 – 50 % of its initial number after 15 days with both cultivars. On the other hand, numbers of spore-formers decreased but not to that extent, and their final recovered numbers at the 90^{th} day were higher with resistant cultivar than susceptible one, which could be explained by the low availability of nutrients required for their germination.

The high R/S ratios (Figure 2) obtained during the first period of plant age, ranged from 5.8 to 15.2 correlate positively with active plant growth during that period. On the contrary, lower R/S values (6.1-13.6) were obtained during the late period where plant growth is usually slow. This was the case for all microorganisms and being higher with resistant cultivars. These results agree partially with Mohamed (1997) who found that such finding was only true for actinomycetes but not for fungal population during his investigation on tomato plants. Higher R/S values were also reported for all microorganisms, except fungi, by Asran (2001) in the rhizosphere-soil of healthy seedlings of cotton cultivars compared to diseased ones. These differences are more likely due to the complex nature of biological activities that are catalyzed by various macro- and microorganisms existing in rhizosphere-soil. Interactions between rhizospheric microorganisms (Baker and Cook, 1974), nature and quantities of root exudates released by different cultivars at different stages of growth (Mohamed, 1997) and differences in cultivar genetic and its degree of susceptibility (Asran, 2001) could all be considered to explain such discrepancy between investigators.

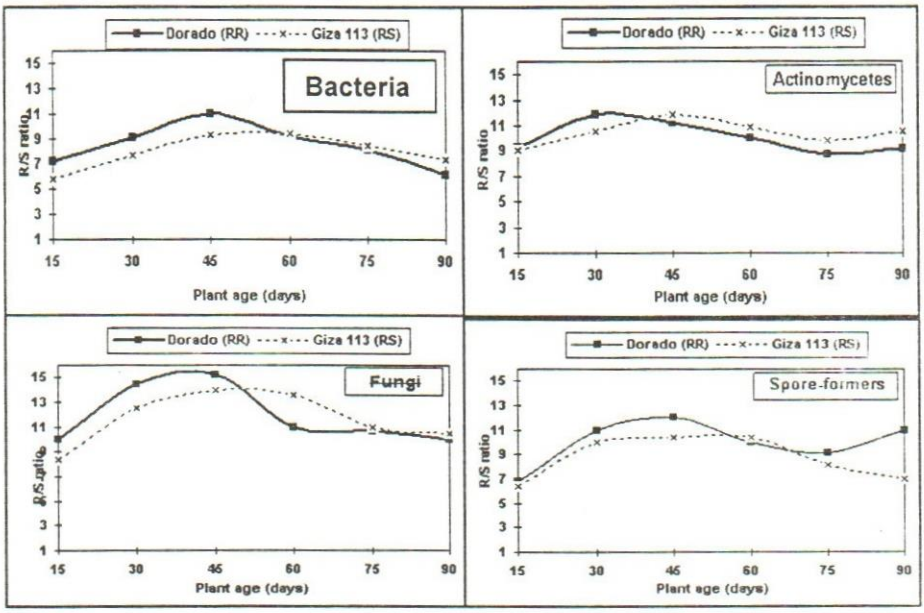

Fig.(2): The ratio (R/S) between counts of microorganisms in the rhizosphere (R) and soil (S) for a resistant and a susceptible grain sorghum cultivars at different growth stages.

Screening of microbial isolates for *in vitro* antibiosis towards *A. strictum*

Results presented in Tables (1 and 2) indicate that out of the total 367 microbial isolates, 183 (48.4 %) isolates exhibited antagonistic effect against *A. strictum*. One hundred fifty one (82.5 %) of the antagonists were bacteria and 32 (17.5 %) were actinomycetes. The isolated fungi (35 isolates) showed no antagonism against *A. strictum* similar to the findings of Ibrahim (1990).

It should be noted that both the highest total counts (Figure 1) and those of antagonists were recorded during the early stages of plant growth. On the other hand, numbers of antagonists were highest in the rhizosphere of resistant cultivars (57.9%) followed by that of susceptible cultivar (29.0) and being the lowest in soil apart (13.1%). This trend was true for the antagonistic isolates of bacteria and actinomycetes as well. The reason for that was not accurately elucidated, however, it could be speculated that resistant plants might have excreted certain substances that specifically encouraged antagonistic microorganism(s), but not the causal ones. Another explanation might also be considered. Deterioration of infected roots of susceptible plants might have resulted in a release of organics and/or growth promoting substances that suited the non-antagonistic microorganisms, possibly disease causing agents, allowing them to grow rapidly, surpass and eliminate numbers and even types of other microorganisms. Similar explanations were given by Alabouvette *et.al.* (1993) to describe the interaction between *Fusarium spp.*, the causal agent for Fusarium wilt and its biocontrol agent.

Table 1: Antagonistic effect of different bacterial isolates obtained from soil and rhizosphere of sorghum plants against *Aremonium strictum*.

Source of isolates	Mean diameter of inhibition zone, mm. [a]						Antagonistic isolates	Total isolates	% Antagonistics
	1 - 5	6 – 10	11 – 15	16 – 20	21 – 25	26 - 30			
Rhizosphere of resistant cv.	4	13	18	22	17	12 (60.0)[c]	86	134	64.2
Rhizosphere of susceptible cv.	1	5	10	12	9	6	43	109	39.5
Soil	2	2	4	7	5	2	22	48	45.8
Total	7	20	32	41	31	20 (13.2)[b]	151	291	51.9

a, Values are averages of 4 replicates , b, Percentage of the most antagonistic isolate compared to total antagonistic number. C, Percentage of the most antagonistic isolates found in the rhizosphere of resistant plant.

Results showed also that rhizosphere of resistant plants harboured higher number of each microbial group compared to that of susceptible plants or soil apart. For instance, twenty (13.2%) out of antagonistic bacterial isolates were the most effective against *A. strictum* showing inhibition zones ranged from 26 to 30 mm in diameter and 60% of them were recovered from the rhizosphere of Dorado cultivar. Similarly, the most effective actinomycetal isolates represented 28.1% of their total antagonistic number, with 55.6% of them being recovered from rhizosphere of Dorado cultivar (Table 2),but with inhibition zones of much less diameters compared to those developed by bacterial most effective isolates which prove the superiority of the latter isolates. The obtained results are in harmony with those reported by Habib (1979) regarding numbers of antagonistic actinomycetes being higher in the rhizosphere of less susceptible faba bean and lentil cultivars compared to highly susceptible ones. However, they contradict those of Mohamed (1997) who found that most of the isolated antagonistic microorganisms displayed denser populations in the rhizosphere of susceptible cultivars of faba bean and cucumber compared to resistant ones.

On the other hand, the higher populations of antagonistic bacterial isolates with their higher effectiveness against *A. strictum* compared to Actinomyctes (Tables 1 and 2) might be explained by the competitive ability of bacteria for nutrient uptake from surrounding environment in both types of rhizosphere and soil apart. It is well established that bacteria exhibit shorter generation time and faster growth rates compared to actinomycetes and fungi (Caldwell, 1995). Certainly, this demonstrates the crucial role of the screening procedure(s) in selecting a proper potent candidate not only for their antagonistic activities but also for their fast growth rate in order to dominate and express their potentialities. Competition seems the most prevalent among different mechanisms for the biocontrol of soil-borne pathogens (Fravel and Keinanth, 1991).

Table 2: Antagonistic effect of different isolates of actinomycetes isolated from soil and rhizosphere of grain sorghum plants against *A strictum*

Source of isolates	Mean diameter of inhibition zone, (mm.) [a]						Antagonistic isolates	Total isolates	% Antgonistics
	1 - 5	6 – 10	11 – 15	16 – 20	21 – 25	26 - 30			
Rhizosphere of resistant cv.	3	7	5	5 (55.6)[c]	-	-	20	30	66.6
Rhizosphere of susceptible cv.	3	3	1	3	-	-	10	15	66.6
Soil	1	0	0	1	-	-	2	6	33.3
Total	7	10	6	9 (28.1)[b]	-	-	32	51	62.8

a. Values are averages of 4 replicates. b, Percentage of the most antagonistic isolates compared to total antagonistic number. c, Percentage of the most antagonistic isolates found in the rhizosphere of resistant plant.

Identification of the most active isolates

Cultural, morphological and physiological tests were conducted for the twenty bacterial isolates having the most antagonistic activities and for the nine most effective actinomycetal isolates as described by Barnett (1960) and Buchanan and Gibbons (1974). The most effective isolates were considered as *Bacillus* spp. and *Streptomyces* spp., respectively.

The antagonistic activities of the most active *Bacillus* spp. and *Streptomyces* spp. were double-checked using the same technique of inhibition zone against *A. strictum*. Table (3) shows that isolates Nos. (5) and (9) among *Bacillus* spp. isolates were the most potent, inducing the widest inhibition zones of 28.2 and 27.9 mm in diameter, respectively. It was also observed that *Streptomyces* spp. isolates Nos. (3), (4) and (8) showed the highest values of antagonism against *A. strictum* with inhibition zones of 19.1, 18.9 and 18.8 mm in diameter, respectively. Therefore, isolate No. (9) of *Bacillus* spp. and isolate No. (3) of *Streptomyces* spp., obtained from rhizosohere of resistant cultivar, were selected as the most efficient bioagents. *Bacillus* spp. isolate No. (9) was further identified as *Bacillus subtilis*.

Effect of culture filtrates of effective isolates against *A. strictum*

Results (Table 4) revealed that filtrates of both *B. subtilis* (isolate No.9) and *Streptomyces* spp. (isolates No.3) individually or in combination showed different antagonistic reactions against *A. strictum*, depending on type of the antagonist and dilution rate of the filtrate. The antagonistic effect decreased with the more diluted culture by filtrates, with the mixture of the two filtrates showing the highest antagonistic effect. The maximum inhibition zones of 30.2, 25.3 and 16.5 mm were recorded for the 1:1 mixture and its broth diluted 1:3 and 1:1 preparations, respectively. The second highest antagonistic effect was

achieved by *B. subtilis* preparation whereas *Streptomyces* spp. displayed the lowest. Several investigators have reported *Bacillus* spp. and *Streptomyces* spp.as biocontrol agents against various plant diseases causal microorganisms. Gotta and Tamietti (1990) found that certain metabolites in culture filtrate of *Streptomyces anulatus* maximized the protection of treated tomato plant against Fusarium wilt through induction of host resistance rather than acting directly on the pathogen. On the other hand, several isolates of *Streptomyces* spp. produced antimicrobial compounds that were responsible for growth inhibition of fungal plant pathogens (Tre Jo-Eskarada *et al.*, 1998).Similarly, culture filtrates of the bacterium *B. subtilis* were reported to contain certain antibiotics that inhibited, *in vitro*, the growth of *F. oxysporum* (Kapoor and Kumar, 1991), *F. moniliforme* (Habbar *et al.*, 1992) and *Pythium* sp. (Hwang *et al.*, 1996). In addition, Asaka and Shoda (1996) identified the antibiotics, produced by the *B. subtilis* and was effective in controlling damping-off in tomato, to be Iturin A and Surfactin.

Table 3: Antagonistic effects of the most effective *Bacillus* spp. and *Sterptomyces* spp. isolates against *A. strictum*.

Isolate No.	Antagonist	Source of antagonist	Mean diameter of inhibition zone (mm)[a]
1	*Bacillus* spp.	Rhizosphere of resistant cv. (RRC)	26.8
2			27.1
3			26.7
4			27.3
5			27.9
6			27.3
7			26.1
8			26.5
9			28.2
10			26.2
11			27.3
12			26.1
13		Rhizosphere of susceptibl cv. .(RSC)	26.4
14			26.6
15			27.3
16			26.5
17			27.2
18			26.7
19		Soil	25.8
20			26.3
1	*Streptomyces* spp.	RRC	16.6
2			17.2
3			19.1
4			18.9
5			16.5
6		RSC	16.2
7			17.1
8			18.8
9		Soil	16.7

a, Values are averages of 4 replicates.

Studies on control of Acremonium wilt disease through the biological activities of both *B. subtilis* (isolate No. 3) and *Streptomyces* spp.(isolate No. 9) are underway in order to elucidate for the most applicable formulation(s).

Table 4 : Effect of different preparations of culture filtrates of *Bacillus subtilis* and *Sterptomyces* spp. against *A. strictum* (diffusion cork borer method).

Culture filtrate preparations [a]	Mean diameter of inhibition zone (mm)[b]		
	B. subtilis filtrate	*Streptomyces sp.* filtrate	Mix. of both filtrates (1:1)
Original	24.1	17.4	30.2
1:3 dilution	19.2	13.9	25.3
1:1 dilution	13.5	9.8	16.5

a, prepared as broth : culture filtrate. b,Values are averages of 3 replicates.

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التضاد بين ميكروبات الريزوسفير وفطر أكريمونيوم ستريكتم المسبب للذبول فى الـذرة الرفيعة

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بتقدير أعداد ألكائنات الدقيقة من بكتريا وفطريات وأكتينوميسيتات وبكتريا متجرثمة فى التربة وريزوسفير صنفى الذرة الرفيعة (القابلة للإصابة (جيزة ١١٣) والمقاومة (دورادو) لمرض الذبول الأكريمونيومى) أثناء مراحل النمو المختلفة حتى ٩٠ يوم من الزراعة وجد أن أعداد الكائنات الدقيقة بصفة عامة فى الريزوسفير أعلى بكثير من أعدادها فى التربة البعيدة. وأن البكتريا هى الأكثر تواجداً بين الكائنات الدقيقة سواءاً فى التربة أو الريزوسفير بينما كانت أعداد البكتريا المتجرثمة ضئيلة.كانت أعداد البكتيريا والفطريات والبكتريا المتجرثمة فى ريزوسفير النباتات المقاومة للمرض أكبر من أعدادها فى ريزوسفير النباتات القابلة للإصابة حتى ٤٥ يوم من الزراعة ثم إنعكس هذا الاتجاه حتى ٩٠ يوم. وأظهرت ١٨٣ عزلة (١٥١ بكتيريا و٣٢ أكتينوميستس)القدرة على تضاد فطر أكريمونيوم ستركتم وذلك من بين ٣٦٧ عزلة (٢٩١ بكتيريا ، ٥١ أكتينوميسيس و٢٥ فطر) تم عزلها من التربة ومن ريزوسفير نباتات جيزة ١١٣ ودورادو وذلك تحت ظروف المعمل. كما أظهرت ٢٠ عزلة بكتيريا عرفت على أنها تابعة لجنس باسيلس وكذلك ٩ عزلات أكتينوميسيتس عرفت على أنها من جنس ستربتوميسيس قدرتها الغالية على تثبيط النمو الميسليومى لفطر أكريمونيوم ستركتم. وجد أن العزلة رقم ٩ من البكتيريا والتى عرفت على أنها باسيلس ستلس والعزلة رقم ٣ من الأستربتوميسيس هى أقوى العزلات فعالية ضد فطر أكريمونيوم ستركتم. كما إتضح فعالية راشح مزرعة البكتيريا (باسيلس ساتلس) العزلة رقم ٩ وكذلك عزلة الأستربتوميستس رقم ٣ وخليط منهما بنسبة ١:١ (**كل على حدة**) فى تثبيط نمو ميسليوم أكريمونيوم ستركتم .