ISSN : 0974 - 7532 Volume 9 Issue 12

Research & Reviews in

BioSciences

Regular Paper

RRBS, 9(12), 2014 [418-426]

Combating mango malformation using bioactive components of *Streptomyces aureofaciens*

Wafaa M.Haggag*, Mohamed D.E.H.Aly

Department of Plant Pathology National Research Center, Dokki, Cairo, (EGYPT)

E-mail: wafaa_haggag@yahoo.com

ABSTRACT

Malformation disease of Mango (Mangifera indica L.) caused by *Fusarium moniliforme var. subglutinans* is one of the most destructive diseases, which is a major production constraint in the mango-growing regions of Egypt. We isolated naturally occurring actinomycetes; Streptomyces aureofaciens with an ability to produce metabolites having antifungal property against pathogen. It was found that *S. aureofaciens* signiûcantly checked the growth of *F. moniliforme* var. *subglutinans*. Three foliar spraying with bioactive components of *Streptomyces aureofaciens* exhibited a significantly high protective activity against development of mango malformation disease on three successive seasons and increased fruit yield.

KEYWORDS

Antifungal;
Fusarium subglutinans;
Mango malformation and
Streptomyces aureofaciens.

INTRODUCTION

Mangoes are an important fruit crop in Egypt and all over the world Statistics provided by the Egyptian Ministry of Agriculture and Land Reclamation indicate that a total of 151,000 Fadden (equiv. 63419.310.464 ha) are planted with mango trees, with a total production of 0.596 million ton in Egypt alone. Mango suffers from several diseases at all stages of its life. Malformation is the most notorious malady amongst the animate problems affecting both vegetative and floral parts of mango. Malformation is noticed on seedlings and saplings organs. Malformation is the most threatening disease causing colossal losses every year[11]. It causes 50–80% loss every year[14] and creates gross deformations of the vegetative and ûoraltissues in mango[12]. Mango malformation has been intriguing scientists as to its cause and control for more than 100 years. Mango malformation disease (MMD) occurs in Asia, Africa, and the Americas and was first reported in India in 1891. Currently, the disease has spread where mangos are grown and causes the most severe damage in Egypt[10,6-8]. *Fusarium moniliforme* var. *subglutinans* was isolated from vegetative as well as ûoral malformed tissues[7,8,12]. *Varma et al. (1971) realised the need of systemic fungicides for the curative control of the disease as the causative fungus was located in the cortex-phloem portion. The application of different fungicides has not produced satisfactory results.* Bhatnagar and Beniwal (1977) reported that in nurseries typical bunchy top symptoms can be produced in seedlings by inoculating the fungus through soil. The fungus may be present in the parenchymatous cells of the pith region of the malformed tissues, which indicates the systemic na-

ture of causal pathogen. Today both academic and industrial interest in microorganisms is on the rise. Actinomycetes are useful and suitable source of new bioactive natural products. Actinomycetes are widely distributed group of microorganism in nature and have the capacity to synthesise many biologically active secondary metabolites[19]. Streptomycetes are found in plant rhizosphere and attention has been paid to the possibility that they can protect roots by inhibiting the development of potential fungal pathogens. This may be achieved through by the production of enzymes, which degrade the fungal cell wall, or antifungal compounds[5]. Haggag, Wafaa *et al.,* (2011) found that *Streptomyces aureofaciens* inhibited the growth of *Colletotrichum gloeosporioides* on mango, indicating that growth suppression was due to extracellular antifungal metabolites present in culture filtrate. Isolate highly produced extracellular chitinase and *â*-1,3-glucanase. Thus, this study investigated to study antifungal activity of the cell-free culture filtrate of *Streptomyces aureofaciens* to determine whether the production of the extracellular hydrolytic enzymes is involved in its observed effect and enhance the control effect of malformation diseases under field condition in three successive seasons.

MATERIAL AND METHODS

Organisms and media

Isolation of the pathogen from mango fruit with malformation symptom was performed by tissue transplanting technique using potato dextrose agar (PDA). Stock cultures of *Fusarium moniliforme* var. *subglutinans* was maintained on PDA slants and stored at 4 °C. *Streptomyces aureofaciens* was previously isolated from the root tissues of mango trees by the surface-sterilization technique[9]. Culture was purified by streak plate technique and confirmed by colony morphology and screened for their antifungal activity and optimized for metabolites production[9].

Determination of moniliformin and fumonisin

(a) Monilformin analysis

A 500 µl aliquots of culture filtrate was loaded onto a waters sep-pak®RP-18column pre-conditioned with water. The column was eluted with 2ml of ion-pair solution (990:10mix of 85:15water/ acetonitrile and 100:48:1.1 MKH2PO4 / BU4N^{+}OH[20]. A 100µl aliquot was chromatographed on a Lichrosorb®RP-18 250*4.6 MM HPLC with the ion –pair solution described above. The moniliformin was detected with the same detector as above and concentration evaluated by integration at 230nm. concentrations were determined by reference to calibration curves established with standard provided by P. Scott of Health Canada[13].

(b) Fumonisin analysis

The cultured were filtered as above and analysis was performed on each replicate flask respectively as follows: A1 ml aliquot of filtrate was applied to abondElut Certify Ï® (200 mg, varian) column preconditioned by aspiring methanol (6ml) and water (6ml) under vacuum. The minicolumns were then washed with water (6ml) and methanol (6 ml). Fumonisins were eluted with.1% trifluoroacetic acid (TFA) in methanol (3ml). Fumonisin was quantified by HPLC as follows.

HPLC analysis and method development

The HPLC system consisted of a ternary solvent pump (Gynkotek model 480), auto sampler (Gynkotek Gina 50), decade electrochemical detector with a glassy carbon electrode (Antec) and a diode array detector (Gynkotek340S). Gynko soft software V5.60 was used to control the HPLC system and for data acquisition and analysis. The equipment was supplied by Dionex Softron (Idstein, Germany). Three columns, i.e. Multosphere C18 (3 ìm; 125—ÿ4 mm ID), Phenomenex Synergy MAX-RP C12 80A with TMS end-capping (4 ìm;150—ÿ4.6 mm ID) and Phenomenex Synergi Polar RP (ether linkedphenyl phase with polar end-capping) were tested for the chromatographic separation of the above-mentioned substances. The Multosphere column was purchased from CS, Langer wehe, Germany and Phenomenex, Aschaffenburg, Germany supplied the Phenomenex columns. Peak identity was determined by means of retention time and UV spectra that were recorded for all samples in the range 200–400 nm.

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In vitro antifungal activity of extracellular metabolites in cell-free culture filtrates of *Streptomyces aureofaciens*

To prepare the cell-free culture filtrate, the antagonist was cultured into broth medium and incubated on an incubator shaker (150 rpm) at 28 °C. The fermentation broth was collected during the exponential and stationary phases. Cells were removed by centrifugation at 8,000 rpm for 20 min at 4 °C. Cell-free supernatant was filtered aseptically through a sterile membrane with 0.45-ìm pore size and stored at 4 °C. The growth inhibitory effects of the assay as described previously by Prapagdee *et al*.[18] with some modifications. Minimum Inhibitory Concentration (MIC) of these compounds were determined.

Inhibition assays

Cell-free supernatant fluids of antagonist was used in the deferred antagonism assay. Cell-free supernatant fluids were prepared by centrifuging the culture for 15 minutes. Supernatant fluids was sterilized and 25 ml were aseptically dispensed in 125-ml Erlenmeyer flasks before being inoculated with 0.1 ml of a spore suspension of *Fusarium moniliforme* var. *subglutinans* containing 10^4 CFU/ml. Cultures were incubated at 25 ^{0}C for 10 days and analyzed for moniliformin and fumonisin production.

Effect of cultivation conditions on enzyme production chitinase assay

The reaction mixture contained 1 ml of 0.1% colloidal chitin in sodium acetate buffer (0.05 M, pH 5.2) and 1 ml culture filtrate was incubated at 37ºC for 2 h in a water bath with constant shaking. Suitable substrate and enzyme blanks were included. Chitinase activity was assayed by the colorimetric method of[2]. One unit of chitinase activity was defined as the amount of enzyme, which produces 1 ì mole of N-acetylglucosamine in 1 ml of reaction mixture under standard assay condition.

β1,3-Glucanase activity was assayed by colourimetric method of Nelson (1955). Reaction mixtures were incubated at 37°C for 30 min and were stopped by boiling for 5 min. One unit of B-1,3-glucanase activity was defined as the amount of enzyme that releases 1 ìmol of reducing sugar equivalents (expressed as glucose) per min.

In vivo evaluation of antifungal activity

To determine the efficacy of the antifungal metabolite against pathogen in successive seasons, three field experiment were conducted under natural infested conditions, using the susceptible cultivars i.e. Sadekia (8 yr-old) at Noubaria region, El Behera and Ismailia Sedekia (15 yr-old) Governorates. Two foliar sprayers were applied at 30 d intervals starting from 15 th January (about one month before normal flowering) on mango trees in one year 2011 and determined in the second season of 2012. The bioactive components at active concentration mixed with 0.1% carboxymethyl cellulase (CMC) as sticker was sprayed using a knapsack sprayer. Trees were sprayed till run-off with approximately 2 L of spray solution per tree. Treatments were assigned in a randomized complete block design. Trees sprayed with water and fungicide served as a check treatment. Plots consisting of three mango trees were replicated three times. Irrigation, fertilization and other cultural practices were carried out as recommended. The disease incidence was determined as percentage of infected flowers at 30 days interval during the growing season (March to July).

The yield parameters of the sprayed trees were evaluated at harvesting stage by determining (i) the mean number of fruits (ii) mean fruit weight and (iii) fruit yield.

Statistical analysis

The collected data were evaluated statistically using the software spss for Windows (release 7.5.1, 20 December 1996; SPSS Inc., Chicago, IL). Data were subjected to analyses of variance and treatment mean values were compared by a modified Duncan's multiple test ($P < 0Æ05$).

RESULTS

Antifungal activity

Preliminary screening for antifungal production was done by streak on agar medium (Figure 1). *S. aureofaciens* showed significant activitie against

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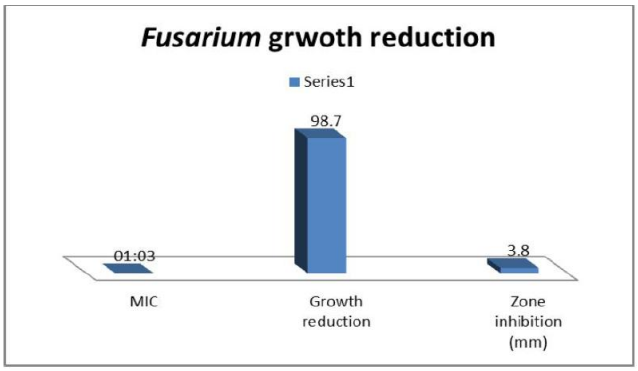

Figure 1 : Antifungal activity of *S. aureofaciens* against *Fusarium moniliforme* var. *subglutinans* growth

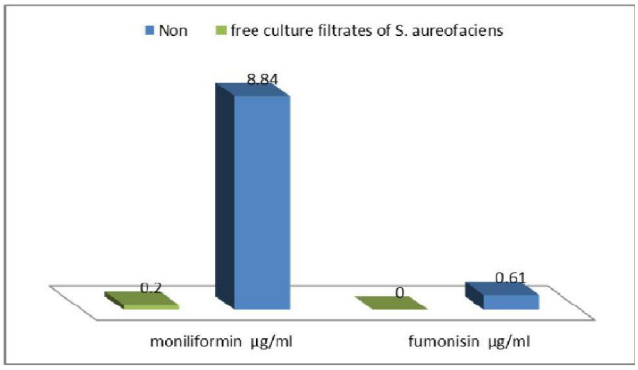

Figure 2 : *In vitro* antifungal activity of cell-free culture of *Streptomyces aureofaciens* on the mycotoxins production by *Fusarium*

pathogen. The antifungal activity of the purified active substance against pathogen was determined. The minimum inhibitory concentration (MIC) was determined by the diffusion method. The nutrient agar plates, seeded with test organisms were used for the MIC determination. The response was observed as a clear zone (mm) around the paper discs (diameter 0.5 mm) loaded with different concentration of active compound (2011) of each concentration were spotted on paper discs. In most cases, purified active substance of *S. aureofaciens* showed antifungal activity against *Fusarium* growth expressed as zone inhibition. The highest reduction was recorded at 1:3 concentration.

In vitro antifungal activity of cell-free culture of *Streptomyces aureofaciens* on the mycotoxins production by *Fusarium*

The ability of *Fusarium* isolate to produce mycotoxins was determined by grown the strains on liquid culture media (Figure 2). Moniliformin and fumonisin were the secondary metabolite mostly produced by *Fusarium* isolate, analyzed using HPLC. High concentration of Moniliformin was obtained in the culture filtrate of *Fusarium* (8.84 moniliformin µg/ml).

Data presented in Figure 2 indicated that cell-free culture of *Streptomyces aureofaciens* isolate inhibited mycotoxins production of *Fusarium moniliforme* var. *subglutinans*. Compelate inhibition was detected of fumonisin. Very low concentration of Moniliformin was detected in the present of cell-free culture of *Streptomyces aureofaciens*.

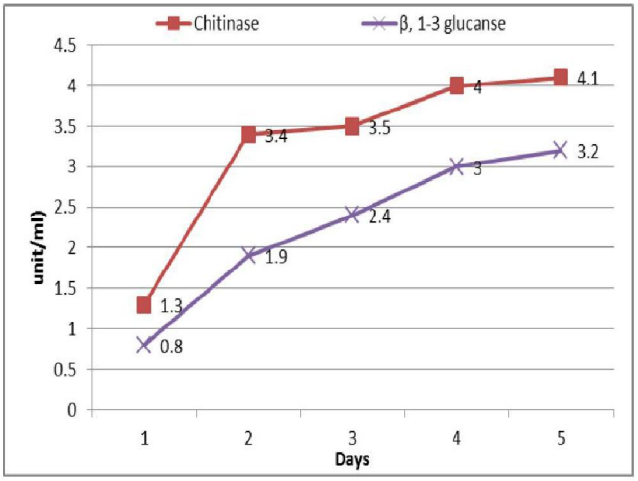

Figure 3 : Hydrolysis enzymes activities in different days

Enzymes assays

The general ability of tested *S. aureofaciens* to produce secondary metabolites include hydrolysis enzymes was determined (Figure 2). Exochitinase and â-1,3- glucanase appeared to be conman metabolites produced by the tested BCAs. Maximum production of chitinase and â-1,3- glucanase by the tested *S. aureofaciens* in shaken broth culture occurred after 150hrs (3.1 Unit| ml).

On field trees

The efficacies of the liquid formulation of *S. aureofaciens* using 0.01% of methylcellulose and tween 80 against mango malformation disease caused by *F. subglutinis* was determined under natural conditions in Ismailia governorate in seasons 2011 and determined in the second season of 2012. Product was applied in a large scale using susceptible cultivars i.e. Ewais, Seddekia, Taimour Zebda and

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TABLE 1 : Efficacy of foliar application of liquid formulation of *Streptomyces aureofaciens* on the first season on mango malformation disease on the second successive season under field conditions

Treatment	Percentage of disease incidence %																			
	Ewais				Saddika				Alphonso				Taimour				Zebda			
	Vegetative		Blossom		Vegetative		Blossom		Vegetative		Blossom		Vegetative		Blossom		Vegetative		Blossom	
	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
Control	53.1	56.4	67.1	58.9	48.6	45.8	61.2	53.8	40.2	45.9	49.4	45.8	38.6	33.6	46.2	45.6	12.2	14.7	16.4	21.7
Streptomyces aureofaciens	0.0	0.0	0.87	0.33	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fungicide	33.4	36.8	43.4	35.7	20.4	13.8	25.2	22.7	24.4	18.6	22.7	18.9	15.4	15.6	20.2	11.5	9.4	12.6	13.5	15.5
LSD	1.2	1.6	2.4	2.6	1.5	1.5	2.6	2.9	1.2	1.9	1.5	1.8	1.7	1.8	1.4	1.6	1.5	1.6	1.7	1.9

TABLE 2 : Efficacy of foliar application of liquid formulation of *Streptomyces aureofaciens* on the first season on mango growth flowering and yield on the second successive season under field conditions

Treatment	Fruit yield | tree																			
	Ewais				Saddika				Alphonso				Taimour				Zebda			
	Fruit number		Yield		Fruit number		Yield		Fruit number		Yield		Fruit number		Yield		Fruit number		Yield	
	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
Control	29.4	33.5	24.3	33.8	26.2	31.7	21.4	26.8	84.5	76.7	53.6	51.6	51.8	43.8	30.4	41.7	62.3	68.7	28.6	31.6
Streptomyces aureofaciens	54.6	65.7	45.7	69.8	52.6	57.8	44.7	47.5	96.8	91.7	72.6	89.6	72.6	69.8	56.4	66.4	81.5	88.7	45.6	51.7
Fungicide (Tobseen)	35.5	41.5	26.4	29.7	31.1	42.6	27.7	31.7	89.4	78.9	60.7	56.5	54.6	49.7	31.5	46.4	64.2	51.4	31.2	33.5
LSD	6.4	7.6	3.5	4.3	4.2	4.6	3.6	4.3	7.8	6.5	6.4	6.1	6.4	5.8	3.6	3.7	5.2	5.7	3.5	3.7

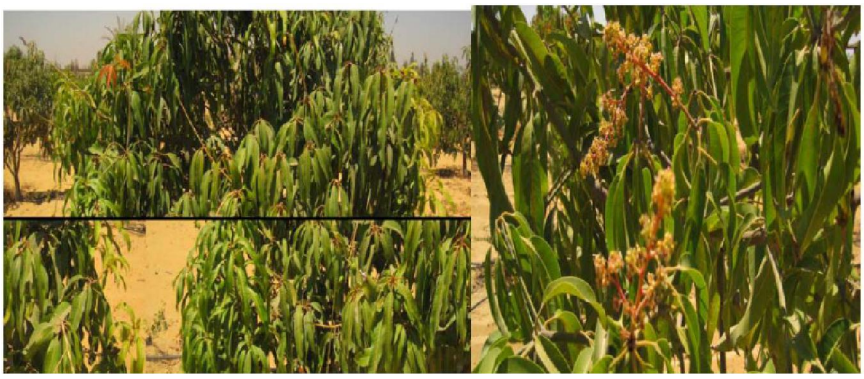
Alphonso TABLE 1. Malformation disease was substantially higher on flowers blossom clusters than in vegetative buds in untreated control in both seasons. The effect of *S. aureofaciens* n fruit numbers and yield was determined (TABLE 2). The reduction in yield was observed under disease infection of all cultivars in both seasons. Application of formulation of *S. aureofaciens* has significantly increased the fruit numbers and yield compared to untreated control and fungicide of all cultivars in both seasons (Figures 4 and 5). *Streptomyces aureofaciens* treatment on the first season reduced mango malformation diseases and increased growth flowering and yield on the second successive season under field conditions (TABLE 1, 2 and Figure 6)

DISCUSSION

In recent years, malformation has become a major challenge to both the pathologists working with the pathogen and to mango researchers in general. Management of malformation can be difficult. New plantings should be established with pathogen-free nursery stock.. Once the disease is found in an orchard, control is possible, but time consuming. In general, the protected, internal location of the pathogen in affected trees makes it difficult to control this disease. Although disease was not completely controlled, this and other systemic fungicides might be useful in future integrated management programmes that would incorporate other measures such as removal of symptomatic terminals and use of tolerant cultivars[7,8].

Fungal phytopathogens pose serious problems worldwide in the cultivation of economically important plants, especially in the subtropical and tropical regions. Despite the undesirable problems caused by the use of synthetic fungicides, fungicides

(A)



(B)

Figure 4 : Efficacy of foliar sprays of different bioagents on the incidence of malformation disease of Awais mango cultivar; A- Control Malformation, B. *Streptomyces* treatment

will be increasingly applied in agriculture in the near future, provided that safer and ecologically friendly fungicides become available. Chemical fungicides not only may pollute the atmosphere but also can be environmentally harmful, as the chemicals spread out in the air and accumulate in the soil. More attention in the last decade has focused on replacing synthetic fungicides with new strategies, such as changes in cultural practices, the use of biological control agents and natural compounds. Interest in biological control of plant pathogens has been stimulated in recent years by trends in agriculture towards greater sustainability and public concern about the use of hazardous pesticides. The desired effects of the use of biological control agents in crop protection have drawn attention to integrated disease management. Biological control agents (BCAs) using antagonistic microbes to reduce the use of chemical fungicides in a system of integrated plant disease management, offers a powerful alternative to control plant diseases. The advantage of using BCAs is that biochemistry and physiology of production of antibiotic antimicrobial substances is well documented. effect of different treatments on the growth of pathogen causing disease.

The current situation is mainly focused on biological control. Since all the commercial mango varieties are susceptible to the disease, biological control provides an effective, persistent and durable protection. *Streptomyces aureofaciens* had the abil-

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Figure 5 : Efficacy of foliar sprays of Streptomyces on fruit yield of Ewais mango cultivar

ity to exhibit high antifungal activity *in vitro* against *Fusarium*. The culture filtrate of this strain had also the ability to *in vivo* suppress infection of *Fusarium* on mango trees. Many species of actinomycetes, especially those belonging to the genus *Streptomyces*, are well known as biocontrol agents that inhibit or lyse several soilborne and airborne plant pathogenic fungi[21]. It is well known that *Streptomyces* sp. can produce industrially useful compounds, notably wide spectrum of antibiotics, as secondary metabolites, and continues to be screened for new bioactive compounds[15]. Spray application of bacterial filtrate on mango trees provided greater efficacy for controlling malformation disease suggested that the bacterial produce some antifungal enzymes for protecting the fruit against the pathogen[4]. This strain is promising for industrial application since they grow quickly in broth condition in simple and of a low cost process to enhance production yield, and the excreted enzymes are frequently required for industrial applications. Therefore, it is thought to be considered as potential industrial candidate for effective saccharification process.

ACKNOWLEDGMENTS

This project was supported financially by the Science and Technology Development Fund (STDF), Egypt, Grant No 216 under title: Development of Bioproducts as Bio-fungicides for Controlling of

Figure 6 : Effect of *Streptomyces aureofaciens* treatment on the first season on mango malformation disease, growth flowering and yield on the second successive season under field conditions

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