Alternative Control Measure for Reducing Citrinin and Alternariol in Broad Beans

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ABSTRACT : An environmental problem was recorded in broad bean crop cultivated in Noubareya region during season 2013. Harvested broad bean seeds were obtained by satisfactory evidence of weak economic value.

An attempt of seed treatment process was carried out in this study to inhibit fungal growth and mycotoxins produced by *Alternaria alternata* and *Penicillium citrinum* in field during cultivation process using sorbic and benzoic acids at 10 and 15 ppm,; ethyl sorbate and/or ethyl benzoate at 5 and10 ppm for each treatment as alternative to the fungicides metalaxyl DS and ridomil MZ72WP at recommended rate (x) and 1 ½ recommended rate applied for seed protection during cultivation process.
The germination test showed the failure of all the tested preservative treatments to germinate seeds. Therefore; all treatments were used as foliar seedlings spray applied 3 times every 15 days during the experiment (60 days) . The results of fungicidal activity indicated that the use of preservative up to 10 ppm totally inhibited fungal growth except ethyl benzoate and ethyl sorbate which completely inhibited fungi at 5ppm Moreover, 5 and 10 ppm concs realized the same inhibition rate exerted by metalaxyl and ridomil MZ at 1.5 x. Untreated plants showed higher death ratios, compared with treated ones. Ridomil MZ at conc. 1.5 x reduced dead plants 60% followed by benzoic acid 56% at conc.5 ppm. Furthermore, the ester form of both benzoic and sorbic acids were more effective in reducing AOH and CTN than the other tested fungicides. metalaxyl and ridomil MZ at 1.5 x. Ridomil MZ at conc. 1.5 x reduced dead plants 60% followed by benzoic acid 56% at conc.5ppm. Furthermore, the ester form of both benzoic and sorbic acids acids were more effective in reducing altrnariol (AOH) and citrinin (CTN) than the other tested fungicides. Accordingly they can act successfully as fungicides alternatives.
Benzoic acid has been actively playing a great role as antifungal and detoxifier agent. Meanwhile, it affected plant growth which badly in need for more studies to avoid these side effects.
Key Words: Alternariol and citrinin detoxification, broad beans, Alternatives to ridomil MZ and metalaxyl, benzoic and sorbic acids.

INTRODUCTION:

Faba bean (*Vicia fabae* L.) is one of the most important food legumes due to its high nutritive value in terms of energy and protein contents (24-30 %),58% carbohydrate and it is an excellent nitrogen fixer (El wakile *et al*., 2009). Egypt is the second largest producer of faba bean in the world, next to china (Torres *et al*., 2006a). The annual faba bean production in Egypt is ranging from 0.56 to 0.58 million metric ton during 2003-2005. However, this production is not enough to meet domestic demand (FLRP, 2004, ICARDA, 2005, Maalouf *et al*.,2009 and yehia *et al* .,2011). The first report concerning a new disease of faba bean crops which has been invaded by *Alternaria tennuisina* in Japan (Mohamed *et al*.,2002 and Barkai-Golan and Nachman,2008).

Alternaria alternata (Fr.) Keissler is a ubiquitous fungi and cause numerous plant diseases and many damages to crops in the field (Mašková *et al*.,2012). This facultative pathogen induces (spots and lesions) mainly on leaves and fruit and less severely on stolons and finaly leads to complete death of the plant. (El Morsi *et al*., 2006 and Vûckovíc *et al*.,2012). This disease was found in several countries and several plant crops (Bodroža-Solarov *et al*.,2012 and Vûckovíc *et al*.,2013). In addition to economic losses in production and processing (Kosiak *et al*., 2004),the disease lead to significant reduction in grain quality (Bodroža-Solarov *et al*., 2012) , this fungus produce AAL toxins (which means *Alternaria alternata* toxins) with different toxicological properties. The presence of these compounds in the food chain is an increasing concern for human and animal health due to their possible harmful effects. (Fern´andez-Cruz *et al*., 2010 and Burkin and Kononenko 2011).

There is a wide range of alternaria toxins but only few of them are associated with health risk acute toxicity (EFSA, 2011). AOH (alternariol) and AME(alternariol monomethyl ether) are of the major concern since they were found to have mutagenic, genotoxic and carcinogenic effects on mammalian cells (Battilani *et al*., 2009 and Bhaat *et al*.,2010).

Alternariol was detected in many agricultural commodities and other foodstuffs (Asam *etal*.,2010 and 2011, Malachova *etal*.,2011,Suchowilska *et al*.,2010, Vučković *et al*.,2012, Eckhout *et al*.,2013).

Both of *Alternaria alternata* and *penicillium citrinum* are not usually broad bean seeds invaders pathogens (Dixit and Singh).According to the available literature, this may be the first registration of this phenomena in Egypt suggesting the possibility to be a new patho-types.

Citrinin is acutely nephrotoxic at relatively high dose in mice and rats, rabbits ,pigs and poultry causing swelling and eventual necrosis of the kidneys and affecting the liver function at a lesser extents (Abou-Zeid ,2012 and Friedman and Rasooly , 2013). There is no specific fungicides for controlling this crop disease case .However,many attempts *in vitro* are reported for inhibiting *Alternaria alternata* and *Penicillium citrinum* using metalaxyl and ridomil MZ (Javaid *et al*.,2006).

Metalaxyl: is used to control certain diseases caused by air-and soil-borne fungi. Foliar sprays with mixture of metalaxyl and protectant fungicides are recommended to control certain air-borne diseases.
Ridomil MZ 72 WP contains metalaxyl 8% and mancozeb 64%. Recently Shu-Yuan Cheng *et al.*, 2014 demonstrated that mancozeb (MZ) induced cytotoxicity in rat pheochromocytoma (PC12) cells partially via nuclear factor kappa B (NF-κB) activation. This study demonstrated that MZ induced DNA damage as seen in comet assay.

Zeidan, (2006) reported that benzoic acid was applied on grapevine in Germanic green houses as MENNO-Florades (9% benzoic acid) to antagonize *Plasmopara viticola*. This application realized highly antagonistic frequency (91.40%), (two days before fungal infection).

Therefore, the aim of this study is to reduce the applied dose of fungicides by using preservatives and investigate their side effects on mycotoxins detoxification under field conditions.

MATERIALS AND METHODS

Fungicides:

Metalaxyl: (IUPAC) name: methyl N-(methoxyacetyl)-N-(2,6-xylyl)-D-alaninate.
Ridomil MZ: methyl N-(methoxyacetyl)-N-(2,6-xylyl)-D-alaninate+ Mancozeb: manganese ethylene bis (dithiocarbamate) (polymeric) complex with zinc salt.

Organic acid used as a food preservatives:

Benzoic: $C_7H_6O_2$ (or C_6H_5COOH).
Sorbic acid, or 2,4-. hexadienoic acid: $C_6H_8O_2$

Structures of mentioned Mycotoxins:

Alternariol is 3,7,9-Ttrihydroxy-1 methyl-6H-dibenzo[b,d] pyran-6-one.Its molecular formula $C_{14}H_{10}O_5$.
Citrinin [C_{13} H_{14} O_{51}] IUPAC: (3R, 4s)-4,6- dihydro-8nhydroxy-3,4,5-trimethyl-6-oxo-3H2- benzopyran-7-carboxylic acid.

The seed borne fungi associated with infected broad bean seeds collected from Noubaria region are previously isolated, purified and identified from surface sterilized seeds using seed health testing method and Frequency of the obtained fungi (*Alternaria alternata ,Penicillium citrinum and Aspergillus flavus*) was determined. Moreover, mycotoxin production ability was estimated and the occurred mycotoxins (alternariol and citrinin) were detected and registered as mentioned in previous study (Youssef *et al*., 2014).

Preparation of chemicals and organic acids serving in Agricultural Experiment:

Benzoic and sorbic acids used as chemical or synthetic preservatives (Class II) are brought from Sigma, Aldrich, Bureau of Cairo Egypt. The food standards regulations required that not more than one Class II preservative should be used in one particular food item (Anand and Sati, 2013). Metalaxyl and ridomil MZ are brought from Syngenta Agro Egypt manufacturing.

Agricultural experiment

Preliminary tests for agricultural experiment:

It was necessary to test the performance of the used tested treatments on seed germination process, and fungal growth. The percent germination process was considered as the determining factor of how tested treatments will be applied during agricultural experiment either seed treatment before seeds Sown process or spraying seedling with the tested treatment every two weeks during the experiment (first test). Furthermore, it was necessary to determine the effect of these treatments on the growth of the occurred fungi associated with infected seeds (second test).

First test: Effects of the tested treatments on seed germination process .

Untreated non infected seeds (Giza blanka) were brought from Research Seeds and Oily seeds Institute, Agricultural Researches Centre, Giza, Egypt. Germination viability test was carried out according to ISTA (1996), Singh and Garampalli, (2012) and Soughir *et al*., 2012 with certain modification. 100 g of seeds were taken from each healthy and/or infected seeds and divided into 5 replicates - 20g each. Two fungicides metalaxyl and ridomil MZ treatments are used at concentrations 0.5 x, x and 1.5 x (where x =500mg /250ml water). Benzoic and sorbic acids and their esters are used at concentrations 5, 10, 15 and 25 ppm. The effect of ethyl alcohol on germination process at concentration 0.25% was tested also. Seeds (20g) in each treatment were placed in sterilized cup 125 ml in volume, the tested concentration was added to sterilized distilled water 100 ml to each cup for each treatment. The negative control for both healthy and infected healthy seed replicates comprise distilled sterilized eater treatment. Each cup solution was decanted 5 hours later, and then weighed. Seeds from each treatment were placed in 120-mm-diameter Petri dishes between two layers of Watman filter paper and then moistened with 10 ml of distilled water for negative control, whereas, the other treatments were moistened with the same solution of the tested treatment . Seeds were kept at room temperature (25°C) under normal light. The number of germinated seeds was counted daily for 7 days after which no further seed germination was occurred. The appearance of 2 mm or more of radical was considered as germination. Parameters measured in this experiment were registered:
Total germination (TG) measured in the seventh day using the formula GT (%) = (total number of germinated seeds (B)/ total seed (A) x 100.(Scott *et al*.,1984 and Moradi *et al*., 2008). The obtained data were registered in Table (1).

Second test 2 :(Confirmation): Testing the treatments effect on growth of fungi occurred in seeds:

The aim of this test was to determine the suitable concentrations for each treatment which will be applied during agriculture experiment. 15 seeds per replicate were taken then soaked in different concentrations of the tested fungicides and preservatives for 10 minutes. Healthy seeds for control were initially surface sterilized using sodium hypochlorite solution 5.0 % for 3 min. and then they were rinsed 3 times with distilled water for 2 min. (Soughir *et al*., 2012). For non infected and naturally infected control, the seeds were soaked in distilled water. Seeds of all the treatments were placed on filter papers in 9 cm diameter petri plates, moistened with 3 ml of distilled water and/or tested treatment concentration each. Plates were incubated in a growth room at 25±2°C for 8days under alternative light and dark 12 hours each. Each treatment was replicated thrice (Christensen, 1957; Javaid *et al*., 2006 and Sitara and Hasan, 2011 with certain modification).

After 8 days of incubation, fungal species grown on the surface of treated seeds were purified and identified and their percentage frequency (PF) was determined according to Samson *et al.* (1996) and Javaid *et al*. (2006). The number of colonies growing on each plate was counted for each identified fungus and its frequency percentage was calculated according to the following formula (Pitt and Hocking, 1997):

PF = (No. of seeds on which fungus appeared / Total No. of seeds) × 100

The resulted data were registered as shown in Table (2). The ability of the main occurred fungi was previously tested and detected as mentioned in precedent study (Youssef *et al*., 2014).The resulted data were registered as shown in Table (2). The selected treatment concentrations for agricultural experiment were summarized in Table (3).

Agricultural experiment:

The clay soil was used in this study ,the electrical conductivity(EC), in decisiveness per meter was measured by Baxter, Digital conductivity meter in saturated soil paste extract as described in hand book 60 (U.S Salinity Laboratory Staff, 1954). Acidity (pH value) was also determined in the tested soil paste using pH meter.

Plastic pots containing 1.5 kg sterilized autoclaved clayey soil for 30 min. and kept covered until sowing seeds. Both of healthy and infected seeds were surface sterilized using sodium hypochlorite 5% for 2 min. Seeds were then rinsed three time in 100 ml sterilized water each to completely get rid of sodium hypochlorite. Weighed seeds for 1hour (10 seeds per pots) were carefully sown. Each treatment was replicated thrice. Pots were placed in complete sunny and fresh air weather to accomplish the field conditions. Seedlings of two weeks age are sprayed with tested treatments. The spraying process was applied and repeated fortnightly**.** At the end of the experiment, the number of dead plants was counted, measured as illustrated in Table (4), plants, 60 days age, were collected, washed then air dried. Plant fresh weight, root and shoot lengths and shoot black lesions lengths (for both dead and alive plants) were measured. Data were registered as illustrated in Table (5).

Estimation of the inhibitory effect of treatments on plant contents of alternariol (AOH) and citrinin (CTN):

The main target of this experiment was to achieve the best treatment which reduce or prevent the production of the two tested mycotoxins during agriculture experiment (*in vivo*) under field conditions. Moreover, this experiment was aimed too to study the following:-The transition of alternariol (AOH) and citrinin (CTN) during the agricultural process from sown seeds to plant; and the effectiveness of the applied spraying method in reducing the two tested mycotoxins.

Plants in each treatment were gently cut into small pieces, and then puted in sterilized cups 10 g each. Treatments were triplicated. Plant samples were prepared to be detected firstly by using TLC (Thin layer chromatography) plate

(Jouany, 2007 and Kütt *et al*., 2010).Two development systems were used for each toxin: Anis: 0.5% p-anisaldehyde in methanol/acetic acid/conc; and sulphuric acid (17:2:1v/v/v) for alternariol and benzene: glacial acetic acid: acetone (5:4:1) v/v/v (Frisvad *et al*. 2007 and Eckhout *et al*., 2013). Under UV light at 254 nm AOH was detected whereas, CTN was detected at 366 nm. After that the detection of alterniol (alternariol) and citrinin was confirmed by using quantitatively HPLC-UV technique according to Azcarat *et al.,* (2008), Asam *et al*. ,(2011) and Brzonkalik *et al*., (2011) at Central Lab, Faculty of Pharmacy, Alexandria University. The obtained citrinin and alternariol standards were brought from Sigma, Aldrich ,Cairo, Egypt. Results were registered in Table (4)**.**

Statistical analysis:

The experiment was laid out in completely randomized design with three replications. The data were subjected to statistical analysis using Costat computer package (CoHort Software, Berkeley, CA, USA). Least significant difference (LSD) using Duncan's Multiple Range test was applied to compare the treatment mean values according to McDonald, (2009).

RESULTS AND DISCUSSION

2. a. Determination of the Electric conductivity and pH in soil used in the experiment.

The applied soil was sterilized before planting and soil electrical conductivity (EC) and pH degree were determined. EC of the soil paste was 3.4 dS/m, whereas soil pH was 7.5.

2. b. Tested treatments effects on seed germination process .

The germination viability of seeds under treatments concentration was tested. Resulted data as illustrated in Table (1) indicated the following: The number of seeds in the same weight varied according to fungal infection. Furthermore, the degree of infection varied between seeds in the same yield. Germination process was affected by fungal infection, the average of germinated seeds attained the highest value at metalaxyl at recommended dose (x) and ridomil MZ at (1/2x).

Retardation in germination was observed during treatment with ethyl alcohol 25%, but it was completely inhibited and delayed in treatments, treated with the tested preservatives and their esters. Fungal infection reduced seed germination rate. These findings were totally in harmony with those reported by Embaby *et al*. (2013) and Perelló and larrán (2013). These results limited the mode of treatments application as seedling spraying method. Both of benzoic and sorbic acids and their esters failed to be applied as seed treatment. Our results are completely in agreement with those of Maouni *et al*. (2002), Sitara and Akhter (2007), Saleem *et al*. (2012) and Sarmamy and Khidir, (2013), who reported that ridomil MZ , ridomil MZ gold and metalaxyl increased seed germination percentages. Moreover, results obtained by benzoic acid and other alternatives were totally in agreement with Sunaina and Singh ,(2014) and indirectly coincided with those of Yadav and Singh (2013), who reported that benzoic acid (BA) decreases the germination ratio of *Triticum aestivum* L. sown in pots containing concentrations (0.5,1and 1.5 mM) of BA in dose dependent manner. Maximum 37.5% reduction in germination was recorded at highest concentration of BA. In our study benzoic, sorbic acids and their esters completely failed in germination process. Differences may be attributed to differences in seeds cultivars, and s the applied method. In our experiment, faba bean seeds were completely emerged in aqueous solution in treatment left for 5 h. before decanting water then placed between two filter papers imbibed with the tested treatment concentration. Thus, the treatment was entirely available for seeds, whereas in Yadav and Singh experiment (2013), seeds were sown in soil containing high concentration of benzoic acid each but undoubtly, the bioavailability of treatment in aqueous media is different than soil because soils can absorb this acid, consequently reduce the treatment bioavailability to seeds. Our findings were also in compatible with those reported by Deepavali and Nilima ,(2012), who reported that aflatoxin decreases germination rate.

Second test*:* Inhibitory Effect of the treatments on fungal growth of seed flora:

The effects of the tested treatments on growth of fungal seed flora were confirmed. Treatments have inhibitory effects against *Alternaria alternata, Penicillium citrinum ,Aspergillus flavus, Penicillium sp* and *Aspergillus niger.* Results were registered as elucidated in Table (2).

Metalaxyl had moderate inhibition against *A. alternata*, but increased the *Penicillium citrinum* growth. Our findings were highly in accordance with those of Cohen, (1981), who found that metalaxyl enhanced the penicillium growth in citrus fruits. Our results were also harmoniously compatible with those of Matheron and Porchas (2000) and Saleem *et al*. (2012), who reported that metalaxyl 15% + copper oxychloride and ridomil MZ (metalalaxyl+mancozeb) exhibited a satisfied inhibitive effect against *A.alternata* and *P.expansum*. According to results obtained in the present investigation and those reported by others, chemicals added to metalaxyl such as copper oxychloride or mancozeb ameliorate augmented the inhibitory effect of these mixture against *Penicillium* than metalaxyl alone.

Agricultural experiment:

2.1. Determination of death rate:

The aim of this test is to determine the effect of fungal infection on plant death and investigate the effects of the tested treatments on reducing death rate. Data in Table (4) illustrated that ridomil MZ at conc. 1.5 x realized the best results in maintained plant life under fungal infection conditions, followed by benzoic acid at 10 ppm. Sorbic acid at conc.10 ppm and its ester form revealed approximately the same action. These findings were partially in harmony with Hawthorne *et al*. (2014), who reported that mancozeb (750-800g/kg) significantly suppressed *Alternaria spp* growth, compared with the other tested fungicides. Similarly, the findings of the same authers may also explain our second finding concerning the worth treatment in maintained plant life enhanced by metalaxyl and ridomil MZ at their recommended dose. This notice may also indicated that these recommended doses were applied only for healthy seed and plant protections but not for actually occurred infection treatment. Death rate in untreated infected plant was extremely high. Our findings are harmoniously agreed with those of Perelló and larrán (2013), who reported that *Alternaria* not only reduces germination and vigor of wheat seed but it also causes seedling blight disease in Argentina (Rajput *et al*., 2005 and Perelló, 2010 a and b) its transmission from seed to seedling of wheat seeds has been done in Argentina. Moreover, every year this seed-borne fungi cause heavy yield loss of the crop.

Table (1): Comparing between the influence of certain fungicides, organic acids and esters on germination process of broad bean seeds

Treatment		T.S.N/W For 5replicate (A)	Tested conc.	Average of Germinated seed number (B)	Day at G start	Total germination T.G. (B/A)x100
Control	Healthy control	98i	0.0	56f	3cd	57.143
	Infected control	138b	0.0	68d	4bc	49.275
	Healthy +alcohol	97i	0.25*	55f	6a	56.701
Inf. seeds	+Metalaxyl	135c	X	120a	3cd	88.89
Infected seeds +Fungicides	+Metalaxyl	130g	½ X	110c	4bc	81.48
	+ Metalaxyl	125h	1½ X	65e	5ab	52.00
	+Ridomil MZ	130g	X	115b	2d	88.46
	+Ridomil MZ	145ef	½ X	120a	3cd	82.76
	+Ridomil MZ	130g	1½ X	115b	2e	88.46
Infected seeds +organic acids +Sorbic acid	+Benzoic acid	132cd	5ppm	0.0g	0ef	0.0
	+Benzoic acid	134de	10ppm	0.0g	0ef	0.0
	+Benzoic acid	130g	15ppm	0.0g	0ef	0.0
	+Benzoic acid	135c	25ppm	0.0g	0ef	0.0
	+Sorbic acid	135c	5ppm	0.0g	0ef	0.0
	+Sorbic acid	130g	10ppm	0.0g	0ef	0.0
	+Sorbic acid	132cd	15ppm	0.0g	0ef	0.0
	+Sorbic acid	132cd	25ppm	0.0g	0ef	0.0
Infected seeds + Esters	+Ethyl benzoate	130g	5ppm	0.0g	0ef	0.0
	+Ethyl benzoate	125h	10ppm	0.0g	0ef	0.0
	+Ethyl benzoate	132cd	15ppm	0.0g	0ef	0.0
	+Ethyl benzoate	145ef	25ppm	0.0g	0ef	0.0
	+Ethyl sorbate	134de	5ppm	0.0g	0ef	0.0
	+Ethyl sorbate	133cd	10ppm	0.0g	0ef	0.0
	+Ethyl sorbate	135c	15ppm	0.0g	0ef	0.0
	+Ethyl sorbate	131fg	25ppm	0.0g	0ef	0.0
	$L.S.D._{0.50}$	1.4297		1.8842	1.2272	

Where:

-T.S.N /W/5replicate= Total seed number in 20g seeds per 1 replicate.

*= ethyl alcohol concentration-

- used = 1: 4 sterilized de-ionized water.

**= The germination process started later than the other treatments.

X=recommended dose for application

(R.D) = 0.5g/250ml water**)**.

G start = day in which seed start to germinate.

H. seeds = non infected seeds.

Inf.seeds=infected seeds.

Table (2): Effect of the tested treatments on fungal inhibition

Treatment	Conc.	Number of colonies formed per 15 seeds for each replicate						Tot.Fr.	Inhib. ER %
		A. alternata		*P.citrinum*		*Other fungi*			
			Freq%		*Freq%*		*Freq%*		
Healthy seeds	0.0	0.0	0.0	0.0	0.0	6+2*	10.67*	10.67	
Infected seeds(I.S)	0.0	58	77.34.	12	16.0	5	6.67**	100.01	0.0
I.S.+Ethyl alcohol	0.25ml	56	74.667	12	16	7	9.334**	99.651	0.448
+metalaxyl	1/2Xg/ml	32	71.12	16	21.33	2.0**	2.667*	95.116	4.979
+ metalaxyl	X gm/ml	8	10.667	10	13.33	2.0**	2.667*	26.664	73.363
+metalaxyl	1½Xgm/ml	5	6.667	4	5.333	0.0	0.0	12.334	87.678
+ridomil MZl	1/2Xgm/ml	7	9.333	10	13.33	0.0	0.0	22.663	77.736
+ridomil MZ	Xgm/ml	5	6.667	7	9.333	0.0	0.0	16.0	84.402
+ridomil MZ	1½ Xgm/ ml	1	1.33	3	4.000	0.0	0.0	5.33	94.675
+benzoic acid	5ppm	0.0	0.0	11	14.66	2**+2*	5.33	19.99	80.029
+benzoic acid	10ppm	0.0	0.0	10	13.33	2**	2.667	15.996	84.019
+benzoic acid	15ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
+benzoic acid	25ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
+sorbic acid	5ppm	0.0	0.0	5.0	6.667	0.0	0.0	6.667	93.34
+sorbic acid	10ppm	0.0	0.0	2.0	2.667	0.0	0.0	2.667	97.34
+sorbic acid	15ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
+sorbic acid	25ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
+ Ethyl benzoate	5ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
+Ethyl benzoate	10ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
+ Ethyl benzoate	15ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
+ Ethyl benzoate	25ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
+ Ethyl sorbate	5ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
+ Ethyl sorbate	10ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
+Ethyl sorbate	15ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
+Ethyl sorbate	25ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0

Where: 8*= 6 colonies of *Penicillium* sp and 2 colonies of *Aspergillus niger* & PF = Frequency percentage of each fungal sp.& 2*+2**= 2**Penicillium* sp colonies+ 2***A.flavus* colonies & ** = *A.flavus* colonies

Our selected treatments are summarized as shown in Table (3)

Table(3): Treatment concentrations applied in the Agricultural experiment.

Treatment	Used Tested concentration	Efficacy ratio %
Metalaxyl	X gm/ml	73.363(R.D)
Metalaxyl	1½Xgm/ml	87.678
Ridomil MZ	Xgm/ml	84.402 (R.D)
Ridomil MZ	1½ Xgm/ ml	94.675
Benzoic acid	10ppm	84.019
Benzoic acid	15ppm	100.0
Sorbic acid	10ppm	97.336
Sorbic acid	15ppm	100.0

Table (4): effect of the tested treatments on reducing plant death:

Treatments	Death rate %	AV.of Number of dead Seedlings(per 18seeds) for each replicate	Reduction ratio in death rate%
Healthy control	11.12	2g	-
Infected control	94.45	17a	-
Metalaxyl (x)	88.89	16a	5.56
Metalaxyl (1.5 x)	88.89	16a	5.56
Ridomil MZ (x)	88.89	16a	5.56
Ridomil MZ (1.5x)	33.34	6f	61.11
Ethyl benzoate (5ppm)	66.67	12bc	27.78
Ethyl benzoate (10ppm)	72.22	13b	22.23
Ethyl Sorbate (5ppm)	50.56	10cd	43.89
Ethyl Sorbate (10ppm)	50.0	9de	44.45
Benzoic acid 10ppm	38.89	7ef	56.0
Benzoic acid 15ppm	50.0	9de	44.45
Sorbic acid 10ppm	50.56	10cd	43.89
Sorbic acid 15 ppm	61.12	11bcd	33.33
L.S.D$_{0.05}$		2.6944	

2.2. Effects of the tested treatments on certain plant growth parameters:

Seedling shoot and root lengths, plant fresh weight, black color lesion (BCL) on faba bean seedling shoots were determined. BCL ratio was calculated for each treatment to assess the effects of the tested treatments on curing infected seedlings. Data in Table (4) illustrated that fungal infection significantly reduced all plant growth parameters and realized the highest BCL values, which indicated the relation between BCL produced by these pathogens and their virulence in field. These findings were in harmony with Yamaji *et al*. (2001); Khan *et al*. (2008) and Hawthorne *et al*. (2014).

Our results concerning the effect on shoot length revealed that Ridomil MZ realized the two highest shoot lengths, followed by ethyl benzoate at conc.5 ppm then ethyl sorbate. Metalaxyl and benzoic acid decreased shoot length These findings were in agreement with Wandrey *et al.* (2004), who mentioned that metalaxyl affected plant growth and plant height only when was applied as spraying. Moreover, these results coincided with those reported by Maffei *et al*. (1999); Yadav and Singh (2013) and Sunaina and Singh (2014), who reported that benzoic acid (BA) significantly decreased plant growth and seed germination proportionally with the increase in concentration.

Concerning root length, ethyl benzoate, ethyl sorbate at 5 ppm and sorbic acid at 10 ppm realized the best same action as ridomil MZ at 1.5X on root length. In contrast to that, metalaxyl, benzoic acid and sorbic acid at 10 ppm achieved the worth results. These results are partially in agreement with Magarey *et al*. (1995 and 1997) and Matheron and Porchas (2000), who mentioned that mancozeb ameliorate root and shoot growth than metalaxyl and were in harmony with those of Fujita and Syono (1996), who reported that the extent of inhibition of root growth by benzoic acid at 10 / µM in pir2-l and pir2-l auxl-7 seedlings was 44% and 19%, respectively as compared with control in absence of benzoic acid (BA) without any inhibition of polar auxin transport (Hertel *et al*., 1969 and 1983). On the other hand, Kim and Roh (2014) reported that BA appeared to promote the growth of tobacco plants. The best growth was obtained on day 50, but the activity of the antioxidative enzyme (glutathione reductase) was inhibited, which suggested that faba bean plant is less resistant to BA than tobacco plants.

Seedling fresh weight determinations :both of ethyl benzoate (5ppm) and sorbic acid (10ppm) realized similar effects as ridomil MZ (1.5x) on seedling fresh weight. Our findings were in agreement with those of Magarey and Bull (1994) and Saleem, (2010), who reported that mancozeb augmented shoot and root dry weight of sugarcane. Furthermore, Magarey and Croft (1995) reported that metalaxyl reduced shoot and root dry weight. Moreover, Magarey and Bull (2003) found that mancozeb caused yield decline in affected soil.

Black color lesions (BCL) attained the highest values in case of infected untreated seedlings and was absent in uninfected seedlings. These results indicated that the existence of this phenomenon was completely correlated to phytopathogenicity and plant diseases. According to many literatures, the seedling infection with *Alternaria alternata* or *Penicillium citrinum* do not produce these large black color lesions but the last one appeared when the double infection with both fungus occurred. Thus, it is suggested the presence of a synergistic effect between the two last mentioned fungi which appeared strongly on BCL production is a sort of virulent phytopathogenisty. Our findings are coincided with those of Perelló *et al*. (2005, 2008 and 2012 and Benssassi *et al*. (2009).

Treatment's effectiveness rank was as the following: ethyl benzoate at 5ppm > benzoic acid at 10pm > ridomil MZ at 1.5 x > sorbic acid at 10ppm then ethyl sorbate at 5ppm. Both of benzoic and sorbic acids at 15 ppm had approximately the same efficiency of metalaxyl x.

2.3.Effect of the tested fungicides and their alternative preservatives on alternariol (AOH) and citrinin (CTN) inhibition in obtained plants after agriculture process.

This experiment was carried out on plants collected and prepared for mycotoxins estimation as mentioned above and data were registered and illustrated as shown in Table (5).

Concerning transition of AOH and CTN during the agriculture process from sown seeds to plant, according to Youssef *et al*. (2014), the average of seeds mycotoxins contents in non-treated seeds were 231.00 µg/gm for AOH and 337.00 µg/g for CTN (Table 6). It was remarked that during agriculture process both of the tested mycotoxins were transmitted from infected seeds to infected plants containing 219.739 and 250.950 µg/g in AOH and CTN, respectively. These findings are coincided with those of Muller and Amend (1997) and Perelló and larrán (2013), who suggested that the decline in mycotoxins degradation in plant was resulted from the exhaustion of nutrients lowering the mycotoxin production rate. Furthermore, our results are compatible with those of Miller *et al*., 1983 and Bhatnagar *et al*. (1991), who hypothesized that the biological degradation of mycotoxins in plant is an enzymatic degradation which considered the plant as source of these enzymes. Lugauskas, (2005) mentioned that AOH and CTN need a sort of fermentation to be degraded.

The efficiency of the tested treatment in inhibiting or reducing AOH and CTN during agriculture process: Ethyl benzoate at conc.10 ppm was the best treatment, where complete inhibition of AOH and CTN production, followed by the concentration 5 ppm then ethyl sorbate at 10 ppm and benzoic acid at 10 and15 ppm, followed by sorbic acid at 15 ppm. Metalaxyl at its recommended dose has a strong inhibitory effects on CTN only but ridomil MZ has a quite effects on both toxins. The inhibition rate was reversely proportional to concentration in ridomil MZ. These findings were reasonably compatible with those of Abd-El Ghany and Tayel (2009), who reported that fungal growth inhibition was sometimes inversely proportional to mycotoxin inhibition .In contrast to that, sorbic acid at 10 ppm has an effective inhibitory effects on CTN and moderately effect on AOH.

The effectiveness of the applied spraying method in reducing the two tested mycotoxins: According to our obtained results, the applied spraying method during this experiment was very satisfactory effective in reducing the two tested mycotoxins. This explained its success in making the pre-harvest crop's yield safety for both human and animal consumption and the plant commodities too.

According to Table (5) and (6) we can notice that in case of BCL, AOH and CTN inhibitions, ethyl benzoate had the inhibitoriest effect. It can be suggested that both mycotoxins are involved in BCL appearance and phytotoxity. Such explanation was in agreement with those of Wang (1948); Nickell and Finlay (1954); White and Truelove (1972); Damodaran *et al*. (1975) and Yamaji *et al*. (2001), who concluded that citrinin at 125 ppm caused collapse of the root structure and tissue browning, and the growth of seedlings was inhibited markedly .

CONCLUSION:

According to the obtained results, benzoic , sorbic acids and their ester forms especially ethyl benzoate are very effective in reducing the production of AOH and CTN by *Alternaria alternate* and *Penicillium citrinum*. The use of ethyl benzoate as alternative to the tested fungicides may realize a big challenge in reducing fungicides used for treatment of infected seedlings as spray. Besides, it reduces the phytotoxic effect of these mycotoxins which lead to diminish the virulence of these pathogens in field.

On the other hand, it was obvious that in order to avoid the failure of benzoic and sorbic acids and ethyl sorbate to realize best growth to the infected seedlings, further studies are needed on treatment of the infected seeds with metalaxyl (x) or ridomil MZ(x) before sowing seeds and reduction of the number of spraying time of seedlings with benzoic or sorbic acids or ethyl sorbate by spraying seedlings each 21 days instead of 15 days.

Table (5): Effect of the tested treatments on certain plant growth parameters:

Tested treatments	A	B	C	D	E	F
Healthy control	10ml St.w	16.95a	8.049a	0.0f	0.000e	-
Infected control	10ml St.w	5.43cd	2.282d	15.4a	0.697a	-
Metalaxyl	X	3.40de	3.177cd	12.90ab	0.462b	33.715
Metalaxyl	1.5X	2.35e	2.767cd	5.01cd	0.289c	58.536
Ridomil	X	7.40c	4.71bc	10.860b	0.203cd	70.875
Ridomil	1.5X	11.9b	6.085ab	4.660cd	0.0840de	87.948
Ethyl benzoate	5ppm	13.6b	6.460ab	3.00de	0.0598e	91.42
Ethyl benzoate	10ppm	6.1cd	4.383bcd	4.700cd	0.118de	83.070
Ethyl Sorbate	5ppm	11.4b	3.770cd	4.800cd	0.105de	84.935
Ethyl Sorbate	10ppm	5.75cd	4.660bc	5.100cd	0.268c	61.549
Benzoic acid	10ppm	6.26cd	3.803cd	1.500ef	0.074de	89.383
Benzoic acid	15ppm	4.6de	3.310cd	5.00cd	0.495b	28.98
Sorbic acid	10ppm	11.05b	6.380ab	4.300cde	0.100de	85.653
Sorbic acid	15ppm	3.94de	2.583cd	6.200c	0.477b	31.563
L.S.D $_{0.05}$		2.4209	1.89645	2.68199	0.11852	

Where:
A=Concentration of tested treatments.
B= Average of one seedling shoot length in cm .
C= Average of Fresh weight ofone seedling
D= Black color lesions(BCL) On shoot in cm .
E= BCL ratio on shoot =black lesions length= Av of lengths of black lesions on treated seedlings/Av of total seedlings lengths
X=R.D= 500mg/250ml water.
1.5 X= R.D + 0.5 R.D=750mg/250ml & Seedling = complete growing plant but before flowering then fruiting stages.

Table (6): Assessment of alternariol (AOH) and citrinin (CTN) in obtained plants after agriculture process and efficacy ratios (E.R %) of treatments:

Treatment	Tested conc.	AOH µg/g	E.R.%	CTN% µg/g	E.R.%
Healthy cont.	0.0	0.00000[l]	-	0.0000[g]	-
Infected cont.	0.0	219.739[a]	-	250.95[a]	-
Metalaxyl	X	142.150[c]	35.309	62.283[d]	85.859
Metalaxyl	1[1]/2X	199.013[b]	9.4320	68.138[c]	72.848
Ridomil MZ	X	86.777[d]	60.509	81.676[b]	67.453
Ridomil MZ	1[1]/2X	45.489[g]	79.299	60.972[d]	75.703
Sorbic acid	10ppm	80.9697[e]	63.152	2.1816[fg]	99.131
Sorbic acid	15ppm	36.708[h]	83.294	5.3810[e]	97.856
Benzoic acid	10ppm	60.6515[f]	72.398	4.4048[ef]	98.245
Benzoic acid	15ppm	35.242[h]	83.962	3.9308[ef]	98.434
Ethyl sorbate	5ppm	39.195[h]	82.163	4.0367[ef]	98.391
Ethyl sorbate	10ppm	24.0079[i]	89.074	4.1931[ef]	98.329
Ethyl benzoate	5ppm	13.0401[j]	94.065	1.5724 [fg]	99.373
Ethyl benzoate	10ppm	6.6337[k]	96.981	1.4413[fg]	99.426
L.S.D $_{0.05}$		3.99945		2.64552	

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الملخص العربى

حل بديل للتحكم فى انتاج كل من السترينين والالترناريول على محصول الفول البلدي

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استهدفت الدراسة عدة نقاط :

اولا : دراسة تطوركل من المرض الفطرى (والحادث لاول مرة فى منطقة النوبارية) وانتاج السمين الفطريين تحت الظروف الحقلية ومدى تأثير هذين السمين الفطريين الالترناريول والسترينين على النبات كمنتج نهائى يصل للسلسلة الغذائية للانسان.

ثانيا: دراسة تأثير المبيدات الميتالاكسيل والريدوميل والتى كانت تستخدم لوقاية البذور والنباتات السليمة من الاصابة الفطرية أثناء الزراعة فى معاملة بذور ونباتات مصابة وتسجيل مدى كفاءة هذه المبيدات فى الحد من الاصابة وتقليل انتاج التوكسينات.

ثالثا: دراسة تأثير كل من حمضى السوربيك والبينزويك والاستر الخاص بكل منهما على كل من النبات والتوكسينات المنتجة أثناء الزراعة ودراسة مدى كفاءة هذه المواد كبدائل امنة لتقليل الجرعات المستخدمة من المبيد الفطرى وكذلك دراسة مدى امكانية الاحلال الكامل لهذه البدائل محل المبيدات المستخدمة .

ولقد توصل البحث إلى النتائج التالية : – تم تطبيق طريقة المعاملة بالرش للبادرات كل 15 يوم لفشل الاحماض واستراتها فى عملية انبات البذور. اثبتت الدراسة وجود علاقة طردية بين السمين الفطريين الالترناريول والسترينين و مدى شراسة وتطور المرض الفطرى بما يوضح ان هذين السمين من السموم السامة للنبات (phytotoxic) بالا ضافة لكونهاسموم عصبية (nephrotoxic) وسموم خلوية (cytotoxic) ومسرطنة ايضا(carcinogenic) للانسان والحيوان على حد سواء .كما اثبتت الدراسة وجود نوع من التعاضد بين الفطرين ومااينتجانه من سموم فى التأثير على حجم المناطق السوداء التى تظهر على النباتات المصابة فقط وتقل بعد المعاملات محل الدراسة وتنعدم فى النباتات السليمة والتى بمقارنتها فى المراجع المختلفة لم تكن بهذه المساحة الكبيرة فى حالة وجود نوع واحد من السموم المختبرة وتنعدم فى حالة الاصابة بسلالة فطرية غير قادرة على انتاج اى من السمين.

أظهرت الدراسة تأثيركل من المبيدات المستخدمة و الاحماض العضوية والاسترالخاص بكل منها على كل من النمو الفطرى وانتاج التوكسين ودلت النتائج على التالى:– انه بالنسبة للمبيدات: بلغت نسبة التثبيط للنمو الفطري الكلى94,675% للريدوميل (x1,5 حيثx = مرة) و84,422% للميتالاكسيل عند نفس التركيز مما يدل على تفوق الريدوميل على الميتالاكسيل عند تركيز (1,5 مرة قدر النسبة المقررة لرش البادرات فى حالة الوقاية) كما قلل الريدوميل(عند نفس التركيز المذكور) نسبة الموت للبادرات بنسبة 61,11% . بالنسبة لحمضى البنزويك والسوربيك والاستر الخاص بكل منهما : أحدث كل من حمض البنزويك والسوربيك تثبيطا للنمو الفطرى بلغت بسبته 100%و 97,36% على التوالى وذلك عند تركيز15 جزء فى المليون بينما حقق كل من ايثايل بنزويت وايثايل سوربيت 100% تثبيطا للنمو الفطرى عند تركيز5 جزء فى المليون كما بلغت نسبة الخفض فى المناطق السوداء على الساق% 91,42 فى حالة ايثايل بنزويت 5 جزء فى المليون تلاها حمض البنزويك 10 جزء فى المليون 89,383 % ثم الريدوميل1.5مرة 87, 948% ثم حمض السوربيك 10 جزء فى المليون 85,653%. أظهرت الدراسة أن الأحماض المستخدمة واستراتها كانت أكثر كفاءة فى القدرة على تثبيط وخفض انتاج التوكسين فى النبات النامى تحت الظروف الحقلية حيث بلغت نسبة خفض السترينين 99,426 % 99,373% بواسطة ايثايل البنزويت على التركيزين 5 و10 جزء فى المليون على التوالى تلاه حمض السوربيك على تركيز 10 جزء فى المليون بنسبة 99,131% أما الميتالاكسيل عند النسبة القررة فبلغت 85,859% تلاهاالريدوميل 1.5مرة للنسبة المقررة 75,703% . فى حالة السم الفطرى الالترناريول كا ن المركب ايثايل بنزويت هو الاكثر كفاءة فى خفض التوكسين على كلا التركيزين المختبرين بواقع 94,065% و96,981% على التركيزين 5 و10 جزء فى المليون على التوالى تلاه الايثايل سوربيت 10جزء فى المليون بواقع89,074 ثم كل من حمضى البنزويك و السوربيك 15جزء فى المليون بفاعلية تثبط 83,962% و83,294% على التوالى. كانت نتائج المبيدات أقل فاعلية من البدائل محل الاختبار فى قدرتها على تثبيط انتاج الالترناريول حيث بلغ اقصى تثبيط للريدوميل 79,292% على تركيز 1.5 مرة قدر النسبة المقررة بينما الميتالاكسيل بلغ 35,309 % عند تركيز النسبة المقررة (x) مما يدل على عدم حساسية الالترناريول للميتالاكسيل .

التوصيات: نوصى باستخدام الميثايل بنزويت كبديل للمبيدات الفطرية فى حالة الرش وذلك بعد اجراء مزيد من الدراسات من قبل المختصين بفسيولوجيا النبات لدراسة مقارنة بين هذه المادة والمبيدات من حيث تأثيرها على الخواص الفسيولوجية و الانتاجية للنبات.