

RESEARCH ARTICLE

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INFLUENCE OF CHEMICAL STRESS OF MICRONUTRIENTS ON VIRULENCE OF SOME SEED-BORNE WILT PATHOGENIC BACTERIA

ABSTRACT:

The chemical stress impact of microfertilizer lanthanum, and essential micronutrients metals namely; boron, copper, iron, manganese, molybdenum, nickel, and zinc, in addition to lithium, magnesium and vanadium (10^2 , 10^4 μM) was studied upon virulence of three spreading wilt seed-borne pathogens; *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, *Pantoea stewartii* subsp. *stewartii*, and *Pseudomonas syringae* pv. *syringae*; the causative agents of wilt disease of dry bean, corn and wheat, respectively. The tolerance of studied pathogens was in the following profile: *C. flaccumfaciens* pv. *flaccumfaciens*, $\text{Mn}^{+2} < \text{Mg}^{+2} < \text{Zn}^{+2} < \text{Mo}^{+2} < \text{B}^{3+} < \text{Li}^+ < \text{Fe}^{+2} < \text{Ni}^{+2} < \text{V}^{+5} < \text{La}^{+3} < \text{Cu}^{+2}$, *P. stewartii* subsp. *stewartii*, $\text{Zn}^{+2} < \text{Mo}^{+2} < \text{Li}^+ < \text{Mn}^{+2} < \text{Mg}^{+2} < \text{Fe}^{+2} < \text{Ni}^{+2} < \text{B}^{3+} < \text{Cu}^{+2} < \text{V}^{+5} < \text{La}^{+3}$, and *Ps. syringae* pv. *syringae*, $\text{Zn}^{+2} < \text{B}^{3+} < \text{Mn}^{+2} < \text{Mo}^{+2} < \text{Li}^+ < \text{Mg}^{+2} < \text{Fe}^{+2} < \text{Ni}^{+2} < \text{Cu}^{+2} < \text{La}^{+3} < \text{V}^{+5}$. The growth decreases with the rise of concentrations (10^4 μM), but 10^2 μM of Li^+ , Mg^{+2} , Mn^{+2} , and Zn^{+2} , stimulated the growth of *C. flaccumfaciens* pv. *flaccumfaciens* and *Ps. syringae* pv. *syringae* insignificantly, while *P. stewartii* subsp. *stewartii* has responded only to Mg^{+2} and V^{+5} in the same concentration. The mean effects of metals were significantly different in response to amino acids synthesis and protein accumulation, where a drastic decline in amino acids content was obtained under treatments of Cu^{+2} and La^{+3} (10^2 & 10^4 μM) while V^{+5} (10^2 μM) had a slight insignificant stimulation of amino acids accumulation upon *C. flaccumfaciens*. The proteome analysis of pathogens subjected to the applied metals, have revealed a significant inhibitory effect with V^{+5} , Cu^{+2} and La^{+3} . The lower concentration (10^2 μM) of other metal ions has induced insignificant inhibitory effects except Mg^{+2} which stimulated the growth of *C. flaccumfaciens*. No generalizations regarding differences between gram negative (*P. stewartii* subsp. *stewartii*, and *Ps. syringae* pv. *syringae*) and gram-positive bacteria (*C. flaccumfaciens* pv. *flaccumfaciens*) for different metal sorption can be made on the basis of our work. However, the conspicuous results of V^{+5} , Cu^{+2} and La^{+3} had a remarkable impact on amino acids synthesis, protein polymerization and consequently the growth rate and tolerance of pathogens. Dry mass of the corn seedlings varied greatly according to single, di- or tri- element consortia applied technique, where the most remarkable influence were obtained from application of the three

elements together. Lanthanum nitrate hexahydrate proved more capability to be an inducer or growth promoter than vanadyl sulfate in the healthy corn seed germination. Application of Cu^{+2} , La^{+3} and V^{+5} consortia-biofilm was the most effective for control of *P. stewartii* subsp. *stewartii* growth in the infected corn seeds.

KEY WORDS:

C. flaccumfaciens, *P. stewartii*, *Ps. syringae*, lanthanum, Heavy metals stress.

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INTRODUCTION:

Plant pathogenic bacteria may reduce seed yield depending upon environmental factors prevailing at the time of the infection, the growth stage of the host crop, and cultivar tolerance, among others. Pathogenic bacteria, unlike innocuous commensal alternate between free living and host associated states. The physico-chemical parameters encountered by the bacteria in these two states are very different and exert different demands and stresses on the bacterial cell (Agrios, 2005). Bacterial pathogens have evolved highly sophisticated mechanisms for sensing external conditions and respond by altering the pattern of gene expression with activation of a set of genes whose products assist in survival and turning

off those the products of which are not necessary in a particular environment (Clements *et al.*, 2001). These sensor-activator systems including metalloproteins allow the bacteria to monitor environmental parameters which distinguish host from external environment and adjust gene expression accordingly, particularly by induction of virulence factors (Parsek *et al.*, 1999). Bacterial virulence factors are required to facilitate colonization, to survive under host defenses, and to permit multiplication inside the host which is influenced by the enzymatic defense system. Particularly well described is the ability to coordinate gene expression in accordance with population density, a process termed quorum sensing (QS) (Eberl, 1999).

The transition metal vanadium (V) has relatively few known biological functions. It is found in marine algae's haloperoxidase enzymes (Butler, 1998). It is also accumulated by ascidians, but its biological function (if any) in these organisms is still mysterious (Lovell *et al.*, 2002). The molybdenum (Mo)-nitrogenase, which is the most common and efficient form of the enzyme, has a Mo cofactor at its active site, but when Mo is not available, some bacteria can express an alternative V-nitrogenase, which uses a V cofactor in place of Mo (Crans *et al.*, 2004). Rare earth elements (REEs) include 17 elements of the 6th period of the 3rd group in the Periodic system with Scandium, Yttrium and lanthanides. These REEs frequently occur together in rare earth minerals and have similarities in ionic radii and physical /chemical activities. Lanthanum (La) is the main components of commercial REEs micro-fertilizer and is widely used worldwide since 1970s (Shyam and Aery, 2011).

Common or dry bean wilt causes serious crop losses worldwide, and its causal agent, the bacterium *C. flaccumfaciens* pv. *flaccumfaciens* (Hedges) Collins and Jones, synonyms *Corynebacterium flaccumfaciens* (Hedges) Dowson, is seed transmitted. Recommendations for managing the disease in the field include the use of pathogen-free seed, crop rotation, and resistant cultivars of dry bean (Camara *et al.*, 2009). The pathogen can be transmitted both within and on the seed; it is very resistant to drying and can remain viable for up to 24 years in seed stored in the laboratory. In the field it has been known to survive in soil for at least two winters between bean crops rotated with wheat (Hall, 1991).

The incidence of *Ps. syringae* pv. *syringae* on wheat is generally sporadic, although yield losses can be devastating when environmental conditions are favorable for epidemic development (Duveiller *et al.*, 1993) in susceptible hosts.

P. stewartii subsp. *stewartii* (Mergaert *et al.* 1993) [syn. *Erwinia stewartii* (Smith)] Dye and hereafter referred as *P. stewartii*] the causal agent of Stewart's bacterial wilt of maize (*Zea mays* L.), is responsible for serious crop losses. It overwinters in mature corn flea beetles (*Chaetocnema pulicaria* Melsh.), which spread the disease. Also, the disease can be seed transmitted at a very low frequency and it spreads systemically through the vascular system (Michener *et al.*, 2002).

In the present study, the impact of lanthanum, and ten essential micronutrients metals was studied upon virulence of three wilt seed-borne pathogens; *C. flaccumfaciens* pv. *flaccumfaciens*, *P. stewartii* subsp. *stewartii*, and *Ps. syringae* pv. *syringae*.

MATERIAL AND METHODS:

Chemicals:

Most chemicals were purchased from Sigma, Aldrich chemicals Co. USA, and ADWIC Egypt. Chemicals and buffers for the enzymatic activities of pathogens were obtained from Bio-Rad Laboratories, N.Y.

Bacterial strains and growth conditions:

Bacterial pathogens used in this study and their sources are shown in table 1. *C. flaccumfaciens* pv. *Flaccumfaciens* strain was maintained on a nutrient broth-yeast extract agar Peptone 3 g, yeast extract 3 g, NaCl 5 g, water 1000 ml (Gab-Allah, 2007). *P. stewartii* subsp. *stewartii* was cultured at 28°C on tryptone-yeast-extract phosphate medium (TYP) consisting of 10 g of Bacto-tryptone, 5 g of yeast extract, 2.2 g of K₂HPO₄·3H₂O, 0.75 g of KH₂PO₄, and 18 g of Bacto-agar per liter of distilled water. Identities of *P. stewartii* strains were confirmed by colonial morphology on TYP, oxidative-fermentative reaction, lack of motility, and pathogenicity tests on the susceptible sweet corn.

Table 1. Bacterial strains

Strain	Relevant characteristic	Ref.
<i>C. flaccumfaciens</i> pv. <i>flaccumfaciens</i>	Wild type; Sm ^r , Rf from <i>Phaseolus vulgaris</i> .	Gab-Allah (2007)
<i>P. stewartii</i> subsp. <i>stewartii</i>	Wild type; Km ^r Amp ^r , from <i>Zea mays</i> L.	This study
<i>Ps. syringae</i> pv. <i>syringae</i>	Spontaneous B ³⁺ enrichment, Tc ^r .	(Dawoud & Mawgoud, 2008)

Ps. pv. syringae was maintained on a modified specific solid medium, which has the following composition: L-proline; 5.0 g, MgSO₄·7H₂O; 0.2 g, K₂ HPO₄; 0.08 g, KH₂PO₄; 0.02 g, MnSO₄; 2.1 g, Dist water 1000 ml (Atlas and Parks, 2001). This medium was modified (supplemented with sterile living wheat seedlings (5% w/v) to restore bacterial pathogenicity), inoculum for further experiments were prepared on liquid media (5 ml 10⁹ cfu/50 ml medium).

The biochemical characteristics of the *Ps. pv. syringae* strain were verified (Dawoud, 2007). Antibiotics were added as necessary to maintain selection for resistance markers, selective antibiotic concentrations were: streptomycin sulfate (Sm) at 1,000 μgml^{-1} , rifampin (Rf) at 100 μgml^{-1} , tetracycline (Tc) at 25 μgml^{-1} , kanamycin (Km) at 100 μgml^{-1} and ampicillin (Amp) at 100 μgml^{-1} .

Screening for metal resistance:

The studied pathovar strains were tested for sensitivity or resistance to 11 different metal salts ranging from microfertilizers as lanthanum to micronutrients as boron, copper, iron, manganese, molybdenum, nickel, and zinc, in addition to macronutrients as lithium, magnesium and vanadium.

Inoculum preparation:

A loopfull of bacterial growth from different strains was inoculated in 50 ml sterile specific liquid medium and allowed to grow on shaker at 30°C till bacterial density reach 10^9 cfu ml^{-1} which then were used as an inocula (Dawoud and Mawgoud, 2008).

250 ml conical flasks containing 50 ml of MM agar; MM is consisted of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; 6.4g, KH_2PO_4 ; 15 g, NaCl; 2.5 g, NH_4Cl ; 5.0 g, MgSO_4 (1M solution); 2 ml, Glucose (20%); 20 ml, CaCl_2 ; 0.1ml and distilled water; 1000 ml, (Sambrook *et al.*, 1989) supplemented with sterile metal salt solutions at different concentrations (10^2 , 10^4 μM), on the basis of medium volume, under aseptic conditions. The cultures were allowed to grow on a shaker at the appropriate temperature (30°C) for each strain, harvested after 48 h, the dry weight was recorded and the rest of the biomass were stored at 20°C until sonicated. The cultural filtrate and bacterial biomass extract were subjected to biochemical analysis. The tested metal salts solutions were: LiCl, MgCl_2 , MnCl_2 , NiSO_4 , CuSO_4 , ZnSO_4 , VOSO_4 , FeSO_4 , NaBO, Na_2MoO_4 , and $\text{LaN}_3\text{O}_9 \cdot 6\text{H}_2\text{O}$. Sterile metal concentrations (in the stock solution) were prepared from (10^2 , 10^4 μM) separately, and were applied depending on solubility and previously published toxicity (Maringoni, 2003). Controls consisted of buffer or water alone.

Growth, proteinome and amino acids analysis:

Fifty ml heavy metals-fortified bacterial specific media were distributed in triplicates of 250 ml conical flasks. The flasks were inoculated with 5 ml 10^9 cfu bacterial suspensions. Flasks were incubated at 30°C (shaking incubator) for 48 hrs. bacterial biomasses were harvested by centrifugation (4000 rpm for 15 min) and kept for either fresh or dry (at 80°C) weight analysis (Bollen, 1972), while proteinome and amino acids were determined as follows:

Proteinome characterization:

The different proteinome fractions were measured by different methods: total soluble protein (Chaney and Marbach, 1962), insoluble protein (Lowry *et al.*, 1951).

Amino acid determination:

Amino acid determination was performed according to the method of Widner and Eggum (1966). Oxidation with performic acid, (to protect methionine and cysteine from destruction during acid hydrolysis), were carried out in closed conical flask for determining all amino acids other than tryptophan. Sample of 20-30 mg., was weighed in the conical flask and 5 ml. of performic acid was added. The flask was closed and placed in ice water bath for 16 h. Sodium metabisulfate was added 25 ml. 6N HC was added to the oxidized mixture. The flask was placed in an oven at 110 °C for 24 hr. It was then opened and all was removed by evaporating the sample to dryness in a rotary evaporator. A suitable volume of sodium citrate buffer (pH 2.20) was added to the dried film of hydrolyzed sample which is ready for analysis. The system used for the analysis was high performance Amino Acid Analyzer, Beckman 7300. Total amino acids were determined and tryptophan content was calculated (Russel, 1944).

Biochemical assay of some cellular enzymes:

Preparation of cell extracts with some modification:

Cells were harvested and washed; the pellets were resuspended in 10 ml phosphate buffer (pH 6.7) and kept in ice cold. Approximately 1 mg of packed cell paste to 2 ml of buffer was used. The cell suspension was sonicated at 400 w over ice for a total of 1 min in 15-25 cycles between which the probe was cooled in ice. The sonicate was then centrifuged at 13,000 rpm (10000 xg) at 3°C for 15 min the pellets was discarded and the supernatant was used for all enzyme assay which were performed spectrophotometrically at room temperature, while cell extracts were always kept on ice. The protein concentration in the cells extract was determined by the method of Bradford (1976). Phenylalkaline ammonia lyase, polyphenol oxidase, peroxidase, pectolytic enzyme, proteolytic enzyme, hydrolytic (cellulase), glutamic oxalic transaminase (GOT), glutamic pyruvic transaminase (GPT) activities were measured as follows:

Phenylalkaline ammonia lyase assay:

Phenylalanine ammonia lyase (PAL) activity was determined by the direct spectrophotometric method described by Pascholati *et al.* (1986), with slight modifications. Conversion of L-phenylalanine to cinammic acid at 37°C was accompanied by reading absorbance at 290 nm every 30s (for five and one half min. after the start of the

reaction). The reaction mixture contained 10 μ l leaf extract and 1000 μ l of a 0.2% phenylalanine solution. The reference cuvette contained 10 μ l extraction buffer and 1000 μ l of the 0.2% phenylalanine solution. PAL activities were determined from a standard curve of cinammic acid vs. absorbance (290 nm) and expressed as n mol cinammic acid $\text{min}^{-1} \text{g}^{-1}$ fresh wt.

Peroxidase assay:

Peroxidase activity was determined at 30°C by a direct spectrophotometric method (Hammerschmidt *et al.*, 1982). The reaction mixture consisted of 10 μ l leaf extract, 2 ml of 0.05M pyrogallol, and 10 μ l of 1% hydrogen peroxide (H_2O_2). The reaction was incubated in a water bath, and absorbance at 420 nm was recorded at 30 s intervals for 5 min. the enzyme activity was expressed as change in the absorbance of the reaction mixture $\text{min}^{-1} \text{g}^{-1}$ of fresh weight.

Polyphenol oxidase assay:

Polyphenol oxidase activity was determined at 30°C by a direct spectrophotometric method; the reaction mixture consisted of 200 μ l of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200 μ l of 0.01 M catechol was added and absorbance at 420 nm was recorded at 30 s intervals for 30 min. The enzyme activity was expressed as change in the absorbance of the reaction mixture $\text{min}^{-1} \text{g}^{-1}$ of fresh weight (Mayer *et al.*, 1965).

Pectinase activity assay:

The reaction mixture contained 0.5 ml of enzyme solution + 0.5 ml of 50 mM sodium acetate buffer (pH 4.3) + 1 mg ml^{-1} pectin). The mixture was incubated in water bath at 50°C for 30 m. The reaction stopped by addition of TCA. Pectinase activity was determined at 575nm. The reducing sugar release was measured by galactouornic acid unit of enzyme activity (μg galactouornic acid. g^{-1} fresh weight. m^{-1}) (Miller, 1959).

Proteolytic enzymes assay:

A proteolytic activity was measured with hemoglobin as substrate according to the method described by Leser and Treutter (2005). One proteolytic unit (U) was defined as the amount of enzyme producing 1 μM glycine. $\text{g}^{-1} \text{min}^{-1}$.

Cellulytic enzyme assay:

It is composed of endoglucanase (endo-1,4-D-glucanase (E.C. 3.2.1.4.), exoglucanase (1, 4- β -D-glucanacellobio hydrolase 3.2.1.9.1.), β -glucosidase. 3.2.1.2.1. Enzyme extract was incubated with 0.5 ml substrate laminarin (1mg ml^{-1} 50 mM Na acetate buffer, pH 45) at 37°C for 10 min + 0.5 ml Nelson reagent (Nelson, 1944) was added, the mixture heated at 100°C for 10 min. After cooling and addition of 0.5 ml arsenomolybdate reagent, absorbance of the

colored product was measured at 540nm. β -1, 3-glucanase activity was expressed as mg glucose mg^{-1} protein min^{-1} (Fink *et al.*, 1988).

Glutamate-oxalacetate transaminase (GOT) assay:

0.2 ml of the extract was quickly mixed with 1 ml phosphate – aspartate substrate – buffer (pH. 7.4). The mixture was incubated for 120 min. at 37°C. The enzymatic reaction was stopped by addition of 1 ml 10^{-3} N 2, 4-dinitrophenyl hydrazine reagent. Meanwhile a parallel blank was carried out where the amount of extract was added after addition of the ketone reagent. All tubes were allowed to stand for 20 min. at room temperature, and then 10 ml 0.4 N sodium hydroxide, were mixed with both the experimental and blank tubes. After 5 min. the optical density of the experimental tubes was read against the blank at 546nm (Tonhazy *et al.*, 1950).

Glutamate-pyruvate transaminase (GPT) assay:

The same procedures previously mentioned for glutamate-oxalacetate transamine was adopted for glutamate-pyruvate transaminase except for substrate-buffer where DL-alanine was used instead of L-aspartate. The reaction was incubated only for 60 min. at 37°C (Reitman and Frankel, 1957).

Inorganic and organic phosphorus (mgm g^{-1} dry mass):

Inorganic phosphorus estimation (Ames, 1966); Borate buffer extract was mixed with ammonium molybdate for 10 min then metol@ reagent was added and kept for 15 min., the optical density was measured at 700 nm. Organic phosphorus was determined by the same method except for using digested plant extract.

Estimation of nucleoprotein pentoses ($\mu\text{g} \text{g}^{-1}$ dry mass):

A known weight of the residue was refluxed for one hour with 5% acetic acid, and then filtered. Ribose and deoxyribose were estimated in the filtrate. In some instances clearing was necessary before estimation. Nucleoprotein ribose was estimated where 2 ml of cleared filtrate were mixed with 1 ml concentrated HCl- FeCl_3 and 0.5 ml alcohol-orcinol reagent (1%). The mixture was kept in boiling water both for 20 min. then cooled. The colored solution was diluted to 5 ml and color intensity was measured photometrically at 660 nm (Schneider, 1945). Nucleoprotein deoxyribose was estimated as the cleared filtrate was acidified with 6N hydrochloric acid and 5% trichloroacetic acid before addition of biphenyl amino reagent and heating for 10 min. in a boiling water bath. After cooling, the color intensity was determined photometrically at 600nm (Clark and Eyzaguirre, 1962).

Organic acid analysis:

The total organic acids were determined (as mM citric acid g^{-1} dry mass) in the plant extract by titration to an end point of pH 7.0 against 0.1 M sodium.

Keto Acid Estimation (mM g^{-1} dry mass):

The Freidman and Haugen method (1943) was used in this investigation. In a Stoppard test tube, 3 ml medium was mixed with 1 ml of freshly prepared 0.2% 2,4 dinitrophenyl hydrazine in 2 N hydrochloric acid. The mixture was thoroughly shaken and kept standing, at room temperature, for 30 min. and 8-10 ml ethyl acetate was added and then the mixture was vigorously shaken, several times, until the aqueous layer was free of color. This was quantitatively pipetted off when the two layers completely separated, and discarded. Six ml of 10% sodium carbonate solution was added to the remaining ethyl acetate extract and the mixture was further shaken until the water layer attained constant yellow tint, whereby the keto acid hydrazone was taken in the carbonate solution. When the two layers separated, 5 ml of the extract were quantitatively transferred to another dry test tube containing 5 ml of 2 N sodium hydroxide solution. The mixture was shaken and kept standing for 15 min. before estimated photometrically at 510 nm.

Phenolic compounds:

One gram of plant leaves were homogenized in 10 ml of 80% methanol and agitated for 15 min., at 70°C (Swain and Hillis, 1959). One ml of the methanolic extract was added to 5 ml of distilled water and 250 μ l of Folin-Ciocalteu reagent, and the solution was kept at 25°C. after 3 min, 1 ml of a saturated solution of Na_2CO_3 and 1 ml of distilled water were added, and the reaction mixture was incubated for 1 h at 25°C., the absorption of the developed blue color was measured spectrophotometrically at 125 nm. The total soluble phenolic content was calculated by comparison with a standard curve obtained from a Folin-Ciocalteu reaction with phenol. Results were expressed as phenol equivalents in $\mu g g^{-1}$ of fresh weight.

Growing and analysis of plants:

Zea mays L. were obtained from Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. These seeds were disinfected soaked in water for overnight and sown in earthen pots (60 x 45 cm & 50 kg soil capacity) containing clean formalin-free sterile sand (fortified with the suggested additives of pathogens and heavy metals (Das *et al.*, 1998). The plants (10 plants/pot)

were regularly supplied with half concentrated solution of modified Hoagland and Knops. The concentrations for each element were N 210 ppm, K 235 ppm, Ca 200 ppm, P 31 ppm, S 64 ppm, Mg 48 ppm, B 0.5 ppm, Fe 1 to 5 ppm, Mn 0.5 ppm, Zn 0.05 ppm, Cu 0.02 ppm, and Mo 0.01 ppm. The pots were kept in fenced area under prevailing climatic conditions (Dawoud, 2007). The disease severity was determined as the percentage of the infected leaf areas to the total plant leaf area (Tesso *et al.*, 2004), samples for analyses were collected at 4 w intervals for the following growth analyses: Total leaf areas (infected & non infected plants), TLA, % diseased leaf area (% DLA) were determined according to (Yehia *et al.*, 1985), the diseased leaf area was excised and measured (Dawoud, 2007):

$$\% \text{ disease severity} = \frac{\text{Diseased leaf area (treated with metal)}}{\text{Diseased leaf area (control without metal)}}$$

Measurement of disease severity:

The extent of leaf spots was measured weekly beginning at the third week from sowing, both directly (in cm^2) and as the number of diseased leaves crossed by the lesions was recorded. The severity of the disease can be identified by measurement of lesion area in relation to plant leaf areas from 10 randomly selected plants (Tesso *et al.*, 2004).

Statistical analysis:

The obtained data were subjected to analysis of variance (ANOVA), the least significant differences were used to compare means of treatments at probability 5% (Snedecor and Cochran, 1980) using Mastate Programme. The least significant differences were used to compare means of treatments.

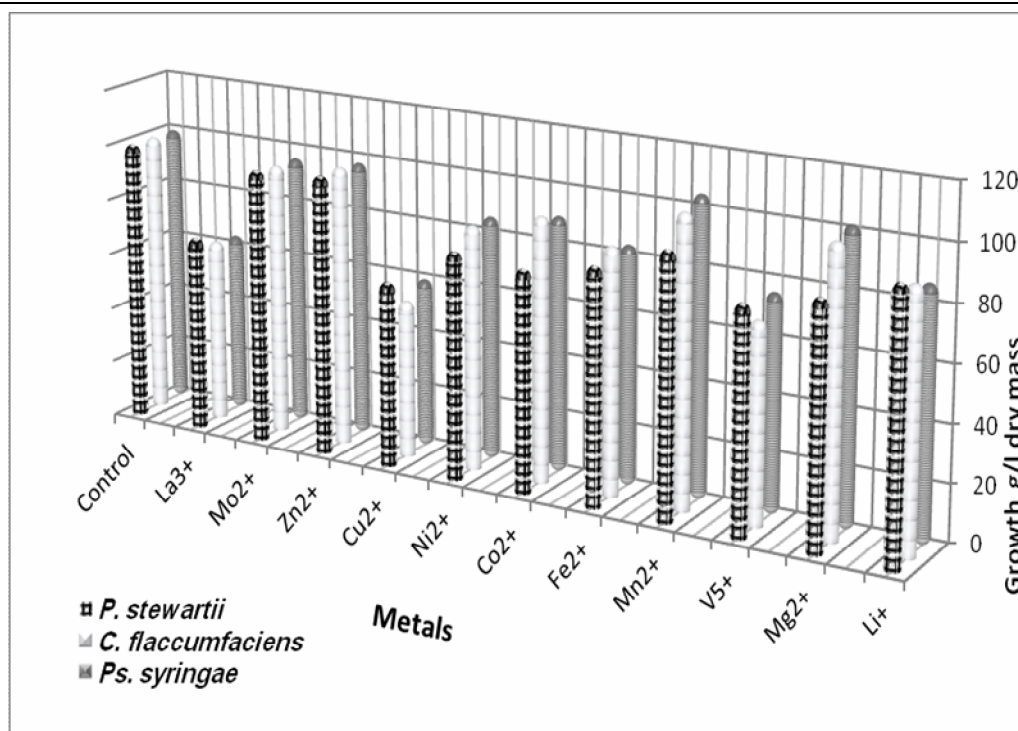
RESULTS AND DISCUSSION:

With the emergence of new pathogens and the increasing antibiotic resistance of old pathogens, novel ways of thinking about therapeutics and for combating infectious diseases were developed. Recent researches are concerned with modern techniques to understand *in vivo* mechanisms of bacterial pathogenesis by studying pathogen-host interactions. By merging the powerful fields of chemical biology and bacterial genomics, a great hope to provide insight into possible new paradigms for addressing infectious diseases was gloried (Gomez *et al.*, 2011).

The results data have shown variation in the response of pathogen's growth in presence of applied metals (Fig.1 & Table 2).

Table 2. Effect of different concentrations of metals salts on pathogens growth (g l^{-1} dry mass). Data with the same letter, at the same column, are not significantly different.

Conc μM	<i>P. stewartii</i>			<i>C. flaccumfaciens</i>			<i>Ps. syringae</i>			
	Mean	% control	Main effect	Mean	% control	Main effect	Mean	% control	Main effect	
Li^+	10^2	1.49	97.38 ^A	93.79 ^A	1.66	100 ^A	90.47 ^A	1.17	94.40 ^B	87.40 ^B
	10^4	1.38	90.20 ^A		1.34	80.95 ^B		1.00	80.40 ^C	
Mg^{2+}	10^2	1.55	101.31 ^A	84.97 ^B	1.74	105.17 ^A	100.17 ^A	1.37	110.62 ^A	102.42 ^A
	10^4	1.05	68.63 ^d		1.58	95.16 ^A		1.17	94.22 ^B	
V^{5+}	10^2	1.59	104.12 ^A	74.94 ^C	1.49	90.00 ^A	70.15 ^C	1.02	82.20 ^C	76.16 ^C
	10^4	0.70	45.75 ^e		0.83	50.30 ^D		0.87	70.12 ^d	
Mn^{2+}	10^2	1.50	98.04 ^A	92.49 ^A	1.70	104.00 ^A	102.00 ^A	1.37	110.42 ^A	105.22 ^A
	10^4	1.33	86.93 ^B		1.66	100.00 ^A		1.24	100.02 ^A	
Fe^{2+}	10^2	1.42	92.81 ^A	83.01 ^B	1.59	96.08 ^A	86.80 ^B	1.12	90.20 ^B	84.12 ^B
	10^4	1.12	73.20 ^C		1.26	76.09 ^C		0.97	78.04 ^d	
Co^{2+}	10^2	1.29	84.00 ^B	78.00 ^{BC}	1.72	104.09 ^A	93.08 ^A	1.25	100.40 ^A	90.12 ^A
	10^4	1.12	72.00 ^C		1.36	82.08 ^B		0.99	80.04 ^C	
Ni^{2+}	10^2	1.32	86.02	80.00 ^B	1.53	92.13 ^A	86.08 ^B	1.14	92.22 ^B	86.15 ^B
	10^4	1.13	73.98 ^C		1.33	80.02 ^B		0.99	80.08 ^C	
Cu^{2+}	10^2	1.50	98.04 ^A	65.36 ^d	1.18	71.10 ^C	55.56 ^D	0.87	70.20 ^d	60.12 ^d
	10^4	0.50	32.68 ^f		0.64	39.01 ^E		0.62	50.04 ^e	
Zn^{2+}	10^2	1.58	103.26 ^A	99.01 ^A	1.81	104.70 ^A	99.41 ^A	1.32	106.50 ^A	98.26 ^A
	10^4	1.45	94.77 ^A		1.56	94.07 ^A		1.12	90.02 ^B	
Mo^{2+}	10^2	1.52	99.35 ^A	97.38 ^A	1.71	102.90 ^A	96.48 ^A	1.28	103.28 ^A	96.48 ^A
	10^4	1.46	95.42 ^A		1.49	90.05 ^A		1.11	89.68 ^B	
La^{3+}	10^2	1.30	84.97 ^B	56.21 ^E	1.50	90.12 ^A	65.07 ^C	0.90	72.20 ^d	64.14 ^d
	10^4	0.42	27.45 ^f		0.66	40.02 ^E		69.5	56.08 ^e	
Control		1.53	100 ^A	100 ^A	166.00	100 ^A	100 ^A	1.24	100 ^A	100 ^A

Fig. 1. Effect of different metals salts on pathogens growth (g l^{-1} dry mass).

The growth decreases with the rise of concentrations, but $10^2 \mu\text{M}$ of Li^+ , Mg^{2+} , Mn^{2+} , and Zn^{2+} , stimulated the growth of *C. flaccumfaciens* pv. *Flaccumfaciens* and *Ps. syringae* pv. *syringae* insignificantly, while *P. stewartii* subsp. *stewartii* has responded only to Mg^{2+} and V^{5+} in the same concentration.

The tolerance of studied pathogens was arranged in the following profile: *C. flaccumfaciens* pv. *Flaccumfaciens*, $\text{Mn}^{2+} < \text{Mg}^{2+} < \text{Zn}^{2+} < \text{Mo}^{2+} < \text{B}^{3+} < \text{Li}^+ < \text{Fe}^{2+} < \text{Ni}^{2+} < \text{V}^{5+} < \text{La}^{3+} < \text{Cu}^{2+}$, *P. stewartii* subsp. *stewartii*, $\text{Zn}^{2+} < \text{Mo}^{2+} < \text{Li}^+ < \text{Mn}^{2+} < \text{Mg}^{2+} < \text{Fe}^{2+} < \text{Ni}^{2+} < \text{B}^{3+} < \text{Cu}^{2+} < \text{V}^{5+} < \text{La}^{3+}$, and *Ps. syringae* pv.

syringae, $Zn^{+2} < B^{3+} < Mn^{+2} < Mo^{+2} < Li^{+} < Mg^{+2} < Fe^{+2} < Ni^{+2} < Cu^{+2} < La^{+3} < V^{+5}$.

The mean effects of metals were significantly different in response to amino acids synthesis and protein accumulation (Table 3 & Fig. 2), where a drastic decline in

amino acids content was obtained under treatments of Cu^{+2} and La^{+3} (10^2 & 10^4 μM) while V^{+5} (10^2 μM) had a slight insignificant stimulation of amino acids accumulation upon *C. flaccumfaciens*.

Table 3. Effect of different concentrations of metals salts on total amino acids content of pathogens (μg g-1 dry mass).

Conc μM	<i>P. stewartii</i>			<i>C. flaccumfaciens</i>			<i>Ps. syringae</i>		
	Mean	% control	Main effect	Mean	% control	Main effect	Mean	% control	Main effect
Li^{+}	10^2	66.32	92.11 ^B	43.18	88.12 ^{AB}	83.15 ^B	68.46	99.22 ^A	90.67 ^B
	10^4	64.17	89.12 ^{BC}	38.31	78.19 ^C	56.65	82.11 ^B	97.01 ^A	88.11 ^{BC}
Mg^{2+}	10^2	69.32	98.06 ^A	47.59	97.12 ^A	88.16 ^{AB}	66.94	97.08 ^A	90.18 ^B
	10^4	61.96	86.05 ^C	38.73	79.04 ^C	54.58	79.10 ^A	83.10 ^B	89.12 ^{BC}
V^{5+}	10^2	66.90	92.92 ^B	51.01	104.10 ^A	99.12 ^A	66.99	97.08 ^A	90.18 ^B
	10^4	57.76	80.22 ^C	46.07	94.02 ^A	57.34	83.10 ^B	92.08 ^A	86.18 ^{BC}
Mn^{2+}	10^2	69.18	96.08 ^A	48.03	98.02 ^A	91.12 ^A	63.54	92.08 ^A	86.18 ^{BC}
	10^4	59.10	82.08 ^C	41.21	84.10 ^B	55.27	80.10 ^B	98.10 ^A	89.12 ^{BC}
Fe^{2+}	10^2	72.06	100.09 ^A	49.05	100.10 ^A	94.16 ^A	67.69	98.10 ^A	89.12 ^{BC}
	10^4	66.26	92.03 ^A	33.33	88.06 ^{AB}	71.30	80.02 ^B	94.16 ^A	89.12 ^{BC}
Co^{2+}	10^2	72.00	100.00 ^A	48.09	98.15 ^A	93.16 ^A	60.00	86.96 ^{AB}	78.99 ^D
	10^4	63.45	88.12 ^{BC}	43.21	88.18 ^{AB}	49.00	71.01 ^C	86.96 ^{AB}	78.99 ^D
Ni^{2+}	10^2	69.93	97.12 ^A	42.19	86.11 ^B	78.11 ^C	65.63	95.12 ^A	90.17 ^B
	10^4	56.90	79.04 ^C	34.30	70.00 ^C	58.86	85.05 ^B	95.12 ^A	90.17 ^B
Cu^{2+}	10^2	49.76	69.11 ^D	60.11 ^E	68.02 ^D	62.12 ^d	52.45	76.02 ^C	68.04 ^E
	10^4	36.72	51.00 ^E	27.49	56.10 ^E	41.41	60.02 ^D	76.02 ^C	68.04 ^E
Zn^{2+}	10^2	64.88	90.11 ^A	85.14 ^C	43.67	89.12 ^{AB}	62.79	91.90 ^A	84.56 ^C
	10^4	57.72	80.16 ^C	34.36	70.12 ^C	79.62 ^C	53.90	78.11 ^C	84.56 ^C
Mo^{2+}	10^2	57.69	80.12 ^C	73.13 ^D	44.14	90.98 ^A	60.62	87.83 ^{AB}	83.15 ^C
	10^4	47.63	66.15 ^D	38.27	78.11 ^C	84.55 ^B	54.14	78.46 ^C	83.15 ^C
La^{3+}	10^2	36.81	51.12 ^E	40.12 ^F	37.78	77.10 ^C	55.28	80.11 ^B	72.11 ^D
	10^4	28.08	39.00 ^F	30.91	63.08 ^D	70.18 ^C	44.16	64.00 ^D	72.11 ^D
Control	72.00	100 ^A	100 ^A	49.00	100 ^A	100 ^A	69.00	100 ^A	100 ^A

Data with the same letter, at the same column, are not significantly different.

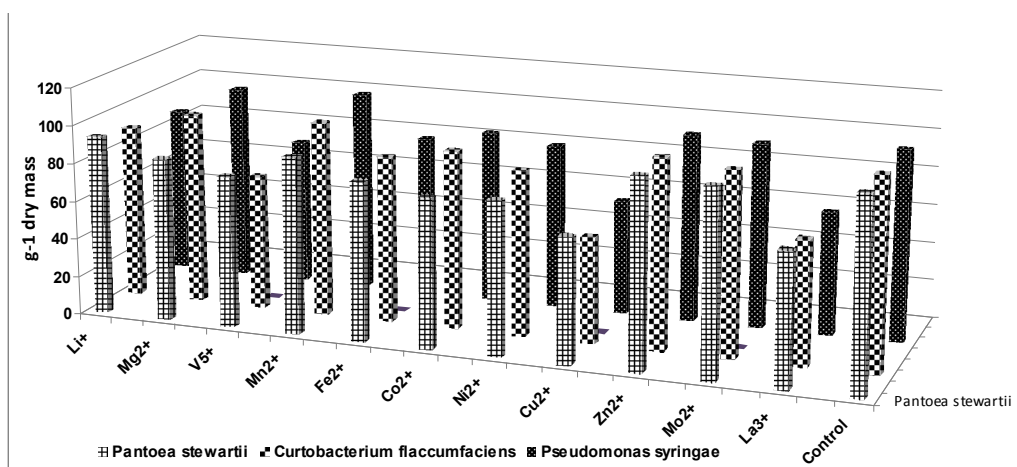


Fig. 2. Effect of different concentrations of metals salts on total amino acid content of pathogens (μg g-1 dry mass)

The proteome analysis of pathogens subjected to the applied metals (Table 4 & Fig. 3), have revealed a significant inhibitory effect with V^{+5} , Cu^{+2} and La^{+3} . The lower concentration (10^2 μM) of other metal ions

has shown insignificant inhibitory effects except Mg^{+2} which stimulated the growth of *C. flaccumfaciens*. No generalizations regarding differences between gram negative (*P. stewartii* subsp. *stewartii*, and *Ps. syringae* pv. *syringae*) and gram-positive bacteria (*C.*

flaccumfaciens pv. *flaccumfaciens*) for impact on amino acids synthesis, protein polymerization and consequently the growth rate and tolerance of pathogens. different metal sorption can be made on the basis of our work. However, the conspicuous results of V^{+5} , Cu^{+2} and La^{+3} had a remarkable

Table 4. Effect of different concentrations of metals salts on the proteinome content of pathogens (mg g-1 dry mass)

Conc μ M	<i>P. stewartii</i>			<i>C. flaccumfaciens</i>			<i>Ps. syringae</i>			
	Mean	% control	Main effect	Mean	% control	Main effect	Mean	% control	Main effect	
Li^+	10^2	0.39	87.12 ^{BC}	84.71 ^C	0.26	78.19 ^{BC}	73.17 ^C	0.44	100.00 ^A	90.06 ^A
	10^4	0.37	82.30 ^C		0.22	68.15 ^C		0.35	80.12 ^B	
Mg^{2+}	10^2	0.43	96.08 ^A	93.16 ^{AB}	0.34	102.21 ^A	98.16 ^A	0.34	98.25 ^A	92.18 ^A
	10^4	0.39	87.08 ^{BC}		0.31	94.12 ^A		0.38	86.11 ^B	
V^{5+}	10^2	0.32	70.11 ^{CD}	60.11 ^D	0.21	64.21 ^C	57.19 ^D	0.33	74.20 ^C	67.16 ^C
	10^4	0.23	50.11 ^F		0.16	50.18 ^C		0.26	60.12 ^D	
Mn^{2+}	10^2	0.45	100.20 ^A	96.18 ^A	0.32	98.14 ^A	89.17 ^{AB}	0.43	98.22 ^A	94.20 ^A
	10^4	0.42	92.18 ^B		0.26	80.21 ^B		0.41	90.18 ^A	
Fe^{2+}	10^2	0.45	100.11 ^A	95.11 ^{AB}	0.32	98.20 ^A	94.12 ^A	0.44	101.2 ^A	99.15 ^A
	10^4	0.41	90.11 ^B		0.31	90.04 ^A		0.42	97.10 ^A	
Co^{2+}	10^2	0.45	98.02 ^A	66.28 ^D	0.33	100.01 ^A	90.06 ^A	0.42	98.00 ^A	89.01 ^A
	10^4	0.16	34.55 ^G		0.26	80.11 ^B		0.35	80.02 ^B	
Ni^{2+}	10^2	0.44	99.17 ^A	91.17 ^B	0.33	99.20 ^A	95.18 ^A	0.41	90.20 ^A	80.11 ^B
	10^4	0.41	93.16 ^{AB}		0.31	91.10 ^A		0.31	70.03 ^C	
Cu^{2+}	10^2	0.31	66.20 ^E	58.16 ^E	0.21	60.22 ^C	49.18 ^D	0.31	70.20 ^C	62.18 ^C
	10^4	0.28	62.12 ^E		0.13	38.14 ^E		0.24	54.16 ^E	
Zn^{2+}	10^2	0.46	102.10 ^A	90.11 ^B	0.27	82.55 ^A	81.37 ^B	0.44	100.01 ^A	95.06 ^A
	10^4	0.35	78.12 ^C		0.25	80.19 ^B		0.39	90.11 ^A	
Mo^{2+}	10^2	0.44	98.12 ^A	94.26 ^{AB}	0.31	92.01 ^A	87.10 ^B	0.43	99.12 ^A	88.67 ^{AB}
	10^4	0.40	90.46 ^B		0.27	82.18 ^B		0.34	78.22 ^C	
La^{3+}	10^2	0.27	60.03 ^E	55.09 ^E	0.21	59.20 ^C	52.11 ^D	0.31	70.18 ^C	65.19 ^C
	10^4	0.23	50.06 ^F		0.15	45.02 ^D		0.26	60.20 ^D	
Control		0.45	100 ^A	100 ^A	0.34	100 ^A	100 ^A	0.44	100 ^A	100 ^A

Data with the same letter, at the same column, are not significantly different.

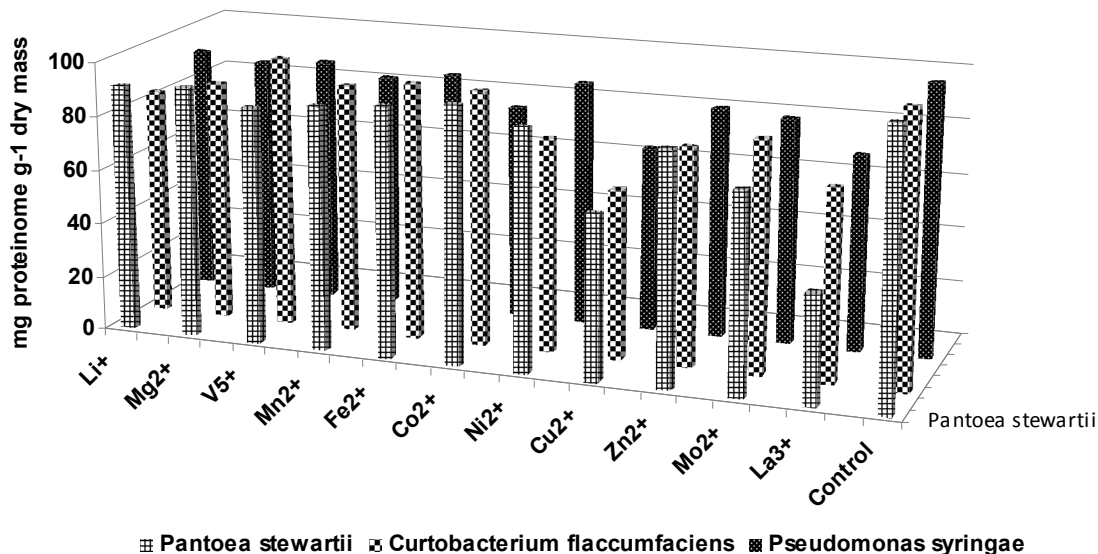


Fig. 3. Effect of different concentrations of metals salts on the proteinome of pathogens (mg g-1 dry mass)

These results may be explained in the discipline that bacteria coordinate expression of functionally related genes to adapt to a rapidly changing in the media constituents of metals. In addition, enzymes which belong to the same metabolic pathway are always upregulated and downregulated together, where gene expression is most frequently modulated at the transcriptional level by the interaction of transcriptional co-factors and metal promoters in bacteria (Lu *et al.*, 2005). Therefore, more consideration have been paid

for V⁵⁺, Cu²⁺ and La³⁺ consortia influence on corn seedlings infected with *P. stewartii* subsp. *stewartii*. Data of influence of Cu²⁺, La³⁺ & V⁵⁺ consortia on seedlings growth of corn seeds (endophytic or biofilmed with *P. stewartii*) and their healthy untreated control seeds (Table 5 & Fig. 4) have revealed that the dry mass of the corn seedlings varied greatly according to single, di- or tri- element consortia applied technique, where the most remarkable influence obtained from application of the three elements together.

Table 5. Effect of different consortia of Cu²⁺, La³⁺ & V⁵⁺ on Seedling growth (g. dry mass) developed from seeds of different treatment with *P. stewartii* and on disease severity.

consortia of Cu ²⁺ , La ³⁺ & V ⁵⁺	Contaminated with endophytic pathogenic bacteria						Healthy seeds coated with pathogenic bacteria					
	Dry mass (g)		Total leaf area (cm ²)		%Disease incidence		Dry mass (g)		Total leaf areas		% Disease incidence	
	M	%	M	%	M	%	M	%	M	%	M	%
Cu ²⁺	1.48 ^C	81.12 ^C	50.80 ^D	79.18 ^D	5.14 ^A	10.12 ^A	1.83 ^D	86.19 ^D	60.72 ^D	84.19 ^D	3.04 ^A	5.0 ^A
La ³⁺	1.90 ^A	104.18 ^A	67.44 ^B	105.11 ^B	1.21 ^C	1.18 ^C	2.50 ^A	118.11 ^A	79.42 ^B	110.12 ^B	0.79 ^B	1.00
V ⁵⁺	1.64 ^B	90.18 ^B	57.21 ^C	89.16 ^C	2.96 ^B	5.18 ^B	1.99 ^C	94.02 ^C	70.76 ^C	98.11 ^C	2.21 ^A	3.13 ^A
% Mean effect	1.67 ^B	91.83 ^B	58.48 ^C	91.15 ^C	3.11 ^B	1.91	2.10 ^B	99.43 ^C	70.30 ^C	97.47 ^C	2.01 ^A	2.71 ^B
Cu ²⁺ +La ³⁺	1.86 ^A	102.0 ^A	64.16 ^B	100.0 ^B	1.20 ^C	1.88 ^C	2.44 ^A	115.11 ^A	79.42 ^B	110.12 ^B	1.68 ^B	2.11 ^B
Cu ²⁺ +V ⁵⁺	1.64 ^B	90.11 ^B	56.54 ^C	88.12 ^C	3.45 ^B	6.11 ^B	2.02 ^C	95.18 ^C	72.20 ^C	100.11 ^C	2.30 ^A	3.18 ^A
La ³⁺ +V ⁵⁺	1.79 ^A	98.18 ^A	64.16 ^B	100.0 ^B	1.29 ^C	2.01 ^C	2.34 ^B	110.16 ^B	72.96 ^C	101.16 ^C	1.37 ^B	1.88 ^B
% Mean effect	1.76 ^A	96.76 ^A	61.62 ^B	96.04 ^C	1.98 ^{BC}	3.33 ^C	2.27 ^B	106.82 ^B	74.86 ^{BC}	103.8 ^C	1.78 ^B	2.39 ^B
Cu ²⁺ +La ³⁺ +V ⁵⁺ Mean effect	1.82 ^A	100.12 ^A	56.56 ^C	88.16 ^C	1.05 ^C	1.86 ^C	2.19 ^B	103.12 ^B	75.09	104.12 ^C	1.58 ^B	2.11 ^B

Data with the same letter, at the same column, are not significantly different.

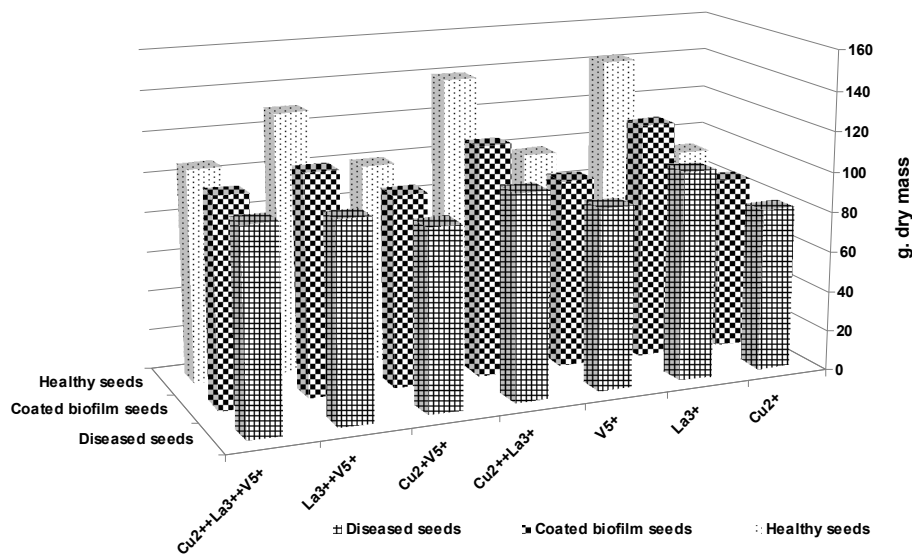


Fig. 4. Effect of different consortia of Cu²⁺, La³⁺ & V⁵⁺ on seedling growth (g. dry mass) developed from seeds of different treatment with *Pantoea stewartii*

In this concern, Ivanov *et al.* (2003) incubated Zea mays seedlings on the solutions of Ag, Cd, Pb, Zn, Cu, Tl, Co, and Hg salts (0.001-3 g/l), and found that for all salts under study, the ratio of the lethal concentration to the lowest concentration slowing down root growth was about ten and the highest metal in toxicity was Cu, related metal affinity of biological compounds for SH-groups with the molar concentration that inhibited primary root growth by 50%, and concluded that the salts under study exert nonselective inhibition and root growth is

slowed down due to the general toxicity of heavy metals rather than selective inhibition of any particular process or processes. Also, Broadhurst *et al.* (2004) found that Plants at 40-mmol kg⁻¹ Ni exhibited the onset of phytotoxicity, and at 60, 80, and 90-mmol kg⁻¹ were demonstrably phytotoxic, but symptoms of phytotoxicity abated within 6 months. The physiological effect of Ni treatment resulted in the decrease in superoxide dismutase and catalase activities in wheat shoots (Gajewska and Sklodowska, 2006), which might favor accumulation of reactive oxygen species in the shoot tissues

and consequently lead to oxidative damage of lipids.

In addition, the same consortia of Cu^{2+} , La^{3+} and V^{5+} were capable to achieve the most impact on growth parameters of *P. stewartii* *in vitro* (Fig. 5). It is probable that some type of post-infection resistance developed, which blocked bacterial colonization in the xylem vessels, preventing

infection of the seeds, (Camara *et al.*, 2009). After *C. flaccumfaciens* pv. *flaccumfaciens*, inoculation of dry bean cultivars with high levels of resistance, observed in xylem vessels agglutinations of bacterial cells surrounded by a structure that apparently blocked further colonization and, consequently, the advance of the disease.

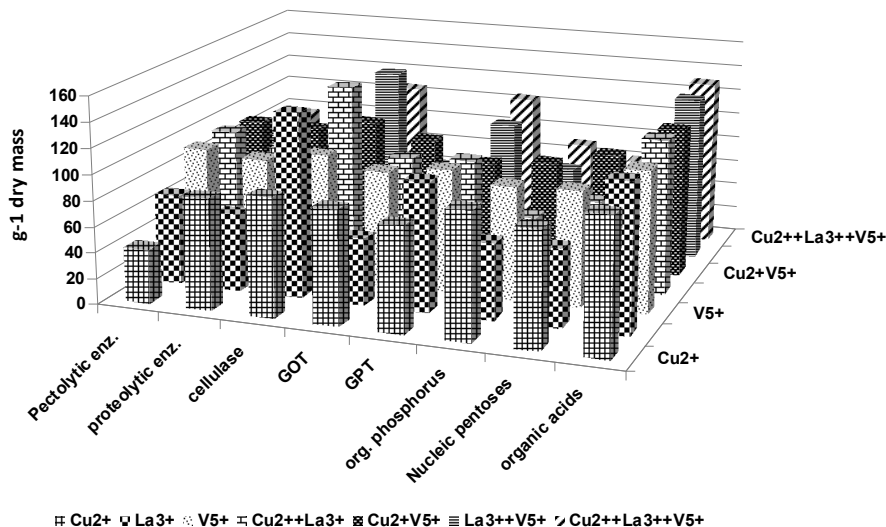


Fig. 5. Effect of different consortia of Cu^{2+} , La^{3+} & V^{5+} on growth parameters of *Pantoea stewartii* *in vitro*

At the same time, lanthanum nitrate hexahydrate proved more capability to be an inducer or growth promoter than vanadyl sulphate in the healthy corn seed germination and that guided us to estimate the proper application of La^{3+} whether in soil treatment, seed soaking or foliar spray (Fig. 6) according to the resulted acquired systemic resistant-inducers of the grown seedlings, where the triple application of soil treatment, seed soaking and foliar spray stimulated the growth radically. This results of La^{3+} promotion for seedlings growth, is in accordance with Chen

et al. (2000) positive effects of La^{3+} on the crop production, such as faster development, greener/dark foliage, larger roots, and better fruit color and quality in different species. Moreover, Wang *et al.* (2003) observed that after applying of La^{3+} , peroxidase activity (POD) in plants increases which resulted in an increase in the environmental stability of the crop against the yield, where POD has been considered as a defensive enzyme and is able to protect the cells from active oxygen damage.

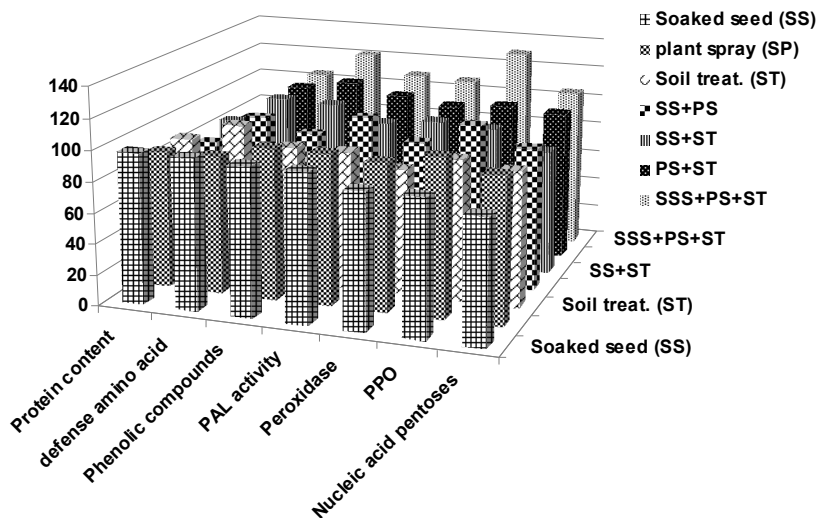


Fig. 6. Effect of La^{3+} on plant metabolites and acquired systemic resistant-inducers of 4 week-seedlings of endophytic seeds

CONCLUSION:

Tolerance of virulent bacteriosis during soaking chemical stress on wilt seed-borne pathovars, namely *C. flaccumfaciens* pv. *Flaccumfaciens*, *P. stewartii* subsp. *Stewartii*, and *Ps. Syringae* pv. *Syringae*; the causative agents of wilt disease of dry bean, corn and

wheat respectively were optimally achieved in the application of Cu^{2+} , La^{3+} , and V^{5+} consortia, where the maximum growth criteria for corn seedlings developed from endophytic grains was tremendously occurred during the application of La^{3+} with the triple application technique of soil treatment, seed soaking and foliar spray together.

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تحمل ضراوة التبكر خلال الإجهاد الكيميائي بالغمر لمسببات الذبول المحمولة بالذور

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تم دراسة الإجهاد الكيميائي لأملح اللانثانيوم كسماد وبعض المعادن المغذية والأساسية للنمو وهي: البورون، النحاس، الحديد، المنجنيز، الموليبدنوم، النيكل، والخصائص بالإضافة إلى الماغنسيوم والفاناديوم وذلك في تأثيرها على ضراوة الأمراض لثلاثة أنواع من البكتيريا المكورة بالذور والمسببة للذبول وهي: كورتوباكتيريوم فلاكومفاسينس المسببة للذبول الفاصوليا الجافة، بانتوي ستيوارتي المسببة للذبول الذرة، و سودوموناس سيرنجي المسببة للذبول القمح. وقد أظهرت النتائج تحمل البكتيريا الممرضة للإجهاد الكيميائي للعناصر على النحو التالي: بالنسبة لنوع كورتوباكتيريوم فلاكومفاسينس. المنجنيز > الماغنسيوم > الخارصين > الموليبدنوم > البورون > الليثيوم > الحديد > النيكل > الفاناديوم > اللانثانيوم > النحاس، وبالنسبة لنوع بانتوي ستيوارتي الخارصين > الموليبدنوم >

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