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Dynamic changes and quantification of defence enzymes activities on chemo-fortified chickpea (*Cicer aritenum* L.) against *Fusarium oxysporum* f. sp. *ciceris*

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ABSTRACT

In pot culture in glass house, we studied the chickpea disease management using synthetic elicitors and defence enzymes against *Fusarium oxysporum* f. sp. *ciceris* [MF803741] on chickpea *cv.* JAKI 9218. Six synthetic chemical elicitors (benzothiadiazole, salicylic acid, potassium silicate, potassium phosphate, humic acid and carbendazim) were used at 3-concentrations. (1.0, 2.0 and 3.0 mM) on test chemo-fortify chickpea *cv.* JAKI 9218. The changes in dynamics of enzyme activity were recorded from upto 24 days from sowing. Among these, salicylic acid fortified at a concentration of 3.0 mM enriched seeds had the lowest incidence of wilt at 17.3 %, 66.0 % reduction in disease and five defence proteins, namely 14, 29, 53, 67, and 83.4 kDa, and four enzyme isoforms. That is, PO1 to PO4 (peroxidase) were expressed; PPO1 to PPO4 (polyphenol oxidases) and three SOD1 to SOD3 (superoxide dismutase). Besides dynamic changes in enzyme activity, peroxidase (absorbance 1.50), polyphenol oxidase (absorbance 1.42), superoxide dismutase (7.60 units), and phenol content (19.24) μg catechol/min/gram tissue increased by 18 days. Hence, using the least quantity of elicitors enhanced and triggered the defence profiles in form of antioxidants, enzymes and proteins in chickpea against to wilt pathogen.

Key words: Antioxidants, benzothiadiazole, chemical elicitors, chickpea, *Cicer arsitemum* L., enzymes, fortification, *Fusarium oxysporum* f. sp. *ciceris*, peroxidase, proteins, wilt.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is major legume crop in the world. It is highly infected by soil-borne wilt disease (10-57 % yield loss) caused by *Fusarium oxysporum* f. sp. *ciceris* (39). Plant disease infections and severity depends on the host age and pathogen virulence (41). The *Fusarium* wilt infects chickpea between stages of ramification to pre-flowering stage (45). When the host is absent, the pathogen, *Fusarium oxysporum* f. sp. *ciceris* remains dormant as chlamydospores from 1-6 years in soil in rest conditions (6,26). When the host is cultivated in the field, it produces mycelial infection peg and penetrates through the root cortex causing the disease (14). Under field conditions, wilt is managed by fungicides, biocontrol agents and resistant cultivars (23). It produces severe ecological changes in cultivated lands and their soil microbiome (8). Traditional practices failed to manage wilt incidence, and pathogen of *Fusarium oxysporum* f. sp. *ciceris* breaks the host resistance barriers / mechanisms with adoptable climatic conditions (35,20). So, triggering the host defence activity from the preliminary stage (seedling stage) may plays a vital role against surveillance, colonization, infection of host and causing wilt

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by *Fusarium oxysporum* f. sp. *ciceris* (7). In recent years, the synthetic chemical elicitors at low molecular concentrations provides effective management of foliar and soil-borne pathogens by immune analogues of salicylic and thousands of R genes (5,29).

Synthetic elicitors are small chemical molecules that can elicit a plant or host immune response by mimicking interactions with plant structure-based defence receptors (33). Hypersensitivity reactions during the infection period cause cell death, a common set of signalling molecules, phytohormones, *viz*., salicylic acid, ethylene and jasmonic acid, regulatory defence proteins and resistant genes were synthesized against pathogens colonization at infection site by systemic acquired resistance (34,42). It triggered the immediate response against the preliminary stage of infection (9). These synthetic chemical elicitors may control the fungal pathogens in crops (19,44). In recent past, several synthetic elicitors (riboflavin, salicylic acid, isonicotinic acid, benzothiadiazole, β-aminobutyric acid, probenazole, paraquat, polyacrylic acid and $HgCl_2$) had been used against several crop pathogens like *Alternaria* spp., *Puccinia* spp., *Erysiphe*, *Sclerotinia* sp., *Botrytis cinerea*, *Colletotrichum* spp., *Xanthomonas* spp., *Fusarium* spp. and *Sclerotium rolfsii* and expressed a set off resistant genes and defence proteins under lab and field conditions (17,30). Keeping this background, the study was carried out for the dynamic changes and quantification of the defence enzymes on chemo-fortified chickpea plants against *Fusarium oxysporum* f. sp. *ciceris* under *in vitro*.

MATERIALS AND METHODS

Experimental treatments

There were 4-experimental treatments: (i). Selection of pathogen and preparation of pathogen inoculum; (ii). Preparation of six synthetic elicitors *viz*., benzo (1,2,3) thiadiazole7-carbothionic acid S-methyl ester (Bion), salicylic acid, potassium silicate, potassium phosphate, humic acid (Lab. Standard) and fungicide - carbendazim (Bavistin) were purchased from commercial sellers. (iii). synthetic elicitors concentrations of treatments 3: 1, 2, 3 Mm (for solid form) and 0.025, 0.05 and 0.1 % for liquid formulations with deionised water and finally recorded the expression of rapid changes in the defence proteins and enzymes at time intervals of 6, 18 and 24 days after treated.

I. Selection of pathogen preparation of pathogen inoculum (sick soil)

A virulent isolate of wilt pathogen (Foc4) *Fusarium oxysporum* f. sp. *ciceris* [MF803741] was selected due to pathogenic ability on chickpea cv. JAKI-9218 under glasshouse conditions. The pathogen isolate (Foc4) was multiplied in sand maize medium. Sand and ground maize seeds were mixed in ratio of 1:4 respectively, moistened with water and sterilized at 121˚C at 15 Pascals for 2 h. The fungus mycelial disc (9 mm) was inoculated into sand maize medium and incubated for 15 days at room temperature (28 ± 2ºC) for multiplication. After multiplying, the inoculum was thoroughly mixed with sterilized pot soil (Red soil: sand: cow dung manure @ 1:1:1 w/w/w) at 10g/kg soil in 15 cm dia plastic pots. Inoculum density was kept at 1×10^6 spores/ml soil and watered for 5 days, depending on soil moisture at sowing (27).

II. Preparation of synthetic elicitors

Six chemical synthetic elicitors (plant defence activators), *viz*., four salt bases: Bion (Benzo (1,2,3) thiadiazole7-carbothioic acid S-methyl ester, SA (salicylic acid), K_2SO_3

(potassium silicate), K_2HPO_4 (potassium phosphate) and two liquid forms of humic acid and carbendazim were prepared at different concentrations, *viz*., 1, 2, 3 mM and the liquid form of elicitors were used at 0.025, 0.05 and 0.1 % in deionised water @ l litre (22).

III. Chemo - fortification

Seeds of chickpea (cv. JAKI-9218) were surface sterilized with sodium hypochlorite solution for 30 secs and rinsed thrice with sterile distilled water. Then, seeds were soaked in prepared synthetic elicitors at 3-concentrations (1.0, 2.0 and 3.0 mM - salt based; 0.025, 0.05 and 0.1 % - liquid form/1 Lit) of above-mentioned synthetic elicitors for 1 h and sown in the sick soil pots. Before sowing, the conidial population was checked at 10^6 conidia / ml in soil-contained pots. In each pot, 3 seeds were sown, and three replicates were maintained. Control pots received only distilled water and pots were kept under the controlled environment (28±2 ºC with >70 % RH). The pots were kept moist and the treatments were replicated thrice in completely randomized block design (15).

IV. Efficacy of chemo-fortification

Data recording: *Fusarium* wilt incidence data was recorded 28 days after sowing and the evaluated on following Scale (21):

I. 1-25 % : Root discoloration or one leaf yellowed,
II. 26-50 % : Root discoloration and more than one leaf yellowed,
III. 51-75 % : Root discoloration plus one leaf wilted,
IV. > 76 % : Root discoloration and plant wilted, and
V. 100 % : Completely dead plant.

The biochemical based induction for the resistance of enzymes was analyzed from the collar region of plant root segments collected at 6, 18 and 24 days after treating. The defence enzymes, *viz*. peroxidase, polyphenol oxidase, superoxide dismutase and phenol content were assessed from the plant sample of 6 (seedlings/5-7 cm of plant height), 18 (ramification I/9-14 cm of plant height) and 24 (ramification II/15-19 cm plant height) (8).

V. Assessment of the dynamic changes in defence enzymes activities

Enzyme extraction

One g root sample was homogenized with 2 ml of 0.1M sodium citrate buffer (pH 5.0) at 4 ºC. The homogenate was centrifuged for 20 min. at 10,000 rpm. The supernatant was used for enzyme activity. Enzyme extracted in 0.1 M sodium phosphate buffer (pH 7.0) was used as the extraction buffer for the assay of peroxidase (PO), and polyphenol oxidase (PPO). Enzyme extract was stored in deep freezer (-70 ºC) until it was used for biochemical analysis (43). After the enzyme was extracted, their defence activities were assessed through methods followed by peroxidase (24), polyphenol oxidase (25), superoxide dismutase (31,18) and phenol (45,32).

Quantification of defence enzymes

After the significantly superior performed conc. of 3.0 mM. Salicylic acid treated plants and their extracts were further used to quantify the defence enzymes with native gel electrophoresis.

(i). Peroxides (PO): To study the expression pattern of different isoforms of peroxidases in different treatments, activity gel electrophoresis was done. For native anionic polyacrylamide gel electrophoresis, resolving gel of 8 % and stacking gel of 4 % were prepared. After electrophoresis, the gels were incubated in the solution containing 0.15 % benzidine in 6 % NH_4Cl for 30 min. in dark. Then, drops of 30 % H_2O_2 were added with constant shaking till the bands appear. After staining, the gel was washed with distilled water and photographed (36).

(ii). Polyphenol oxidase (PPO) : After native electrophoresis, the gel was equilibrated for 30 min in 0.1 % *p*-phenylene diamine in 0.1M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer (28). The addition of catechol was followed by a gentle shaking which resulted in appearance of dark brown discrete bands (13).

(iii). Superoxide dismutase (SOD): Electrophoresis was done under native condition in 8 % polyacrylamide gels for SOD activity staining. Electrophoresis running conditions and gels were prepared with lacking SDS. Equal amounts of protein (40 *µ*g) were loaded on to each lane. SOD activity was determined on native PAGE gels as described by (13) and modified by (2) during barley examination. The gels were rinsed in deionised water and incubated in dark for 30 min at room temperature in an assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium and 0.3 % (v/v) N, N, Ń, Ń-tetramethyl ethylenediamine (TEMED). At the end of this period, the gels were rinsed with deionised water and placed on a light box for 5 to 10 min at room temperature until the development of colourless bands of SOD activity in a purple-stained gel was visible. The reaction was stopped by transferring the gels to 6 % (v/v) acetic acid.

VI. Crude protein analysis - SDS PAGE

One g leaf sample was extracted with one ml of 0.1 M sodium phosphate buffer (pH 7.0) under 4 ºC. The homogenate was centrifuged for 20 min at 10000 rpm and the supernatant was used for the SDS-PAGE (45,12). The protein content of the sample was determined (10). 100 µg of protein from different treatments was taken and mixed with 10 µl of sample buffer in a microfuge tube, boiled for 4 min and incubated at 4 ºC for 30 min. Then the samples containing equal amounts of proteins were loaded into the wells of polyacrylamide gels (Sigma-Aldrich Techware system, Sigma, USA). The medium range molecular weight markers (Bangalore Genei, India) were used, and electrophoresis was carried out at constant voltage of 75 volts for 2 h. The gels were stained with 0.2 % Coomassie brilliant blue (R250) solution. Based on the Rf value of each protein band stained, the molecular weight was calculated.

VII. Estimation of phenols

One g root tissue was homogenized in 10 ml of 80 percent methanol and agitated for 15 min at 70ºC. To 5 ml of distilled water and 250 µl of Folin-ciocalteau reagent (1N), 1 ml of the methanolic extract was added and incubated at 25 ºC for 3 min and after that 1 ml of saturated solution of 20 % sodium carbonate was added and mixed well. Then the tubes were placed in a boiling water bath for 1 min and cooled. The absorption of the developed blue colour was measured at 725 nm and catechol was used as the standard. The total phenol content was expressed in µg of catechol /g of plant tissue (16).

VIII. Statistical analysis

Mean differences of the treatment were evaluated with ANOVA using AgRes statistical package at 5 % significance (Version 3.01, Pascal Intl Software Solutions). All data were statistically analysed with AgRes package and interpreted. Per cent values are transformed with arc sine transformation and other values are transformed with square root transformation for data analysis.

RESULTS AND DISCUSSION

I. Efficacy of chemo-fortification against wilt

All the synthetic elicitors significantly reduced the wilt incidence in chickpea. Among them, 3 mM conc. of salicylic acid recorded the lowest incidence 17.3 % of wilt, followed by 18.3 % compared with carbendazim @ 0.1 % [Bavistin] with disease reduction of 66.0 % and 64.0 % (Figure 1A&1B). These results showed that chemicals like salicylic acid (2 mM) at 30 ml / pot along with *Pseudomonas fluorescens* strain (Pf-92) @ [10^8 spores/ml] treated chickpea plants showed 52-64 % disease reduction in wilt (19). Chemical inducer acibenzolar-S-methyl recorded the maximum disease reduction (79.3 %) on cv. C-44 against *Ascochyta* blight in chickpea at 1.2 mM concentration compared to salicylic acid (21). Commercially available salicylic acid and benzothiadiazole [Bion] (1.0 mM and 0.4 mM) recorded the maximum disease reduction on *Ascochyta* blight of chickpea cv. C727 (30).

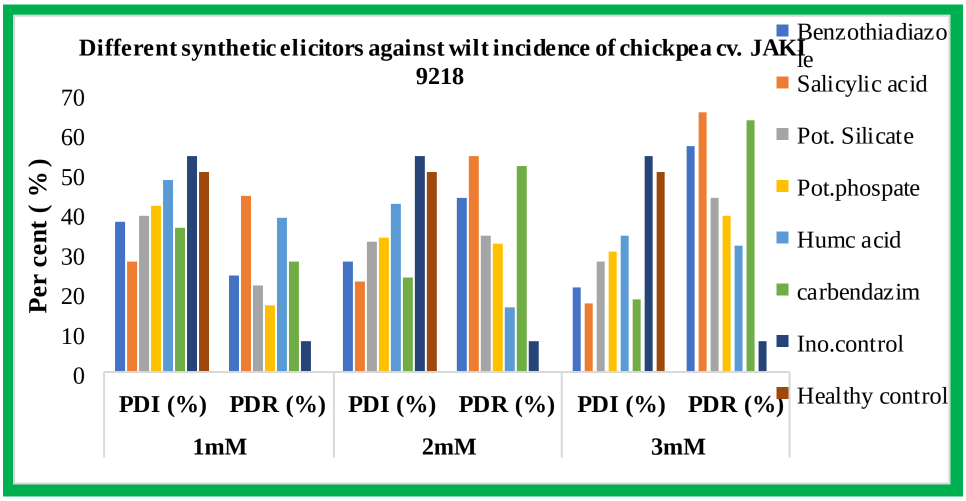


Figure 1A. Efficacy of chemo-fortification of synthetic elicitors against wilt incidence of chickpea cv. JAKI-9218 in pot culture

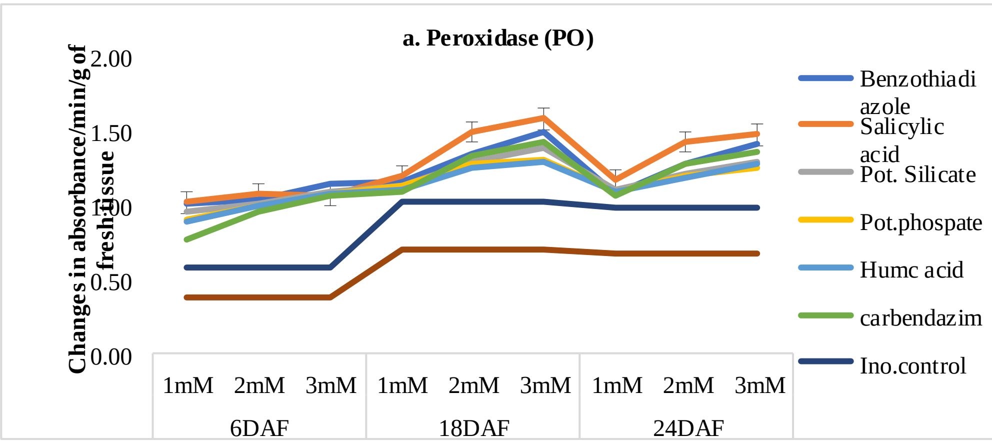
*PDI: Per cent disease incidence, PDR: Per cent disease reduction

a. Overview Under Glasshouse c. (3m M BION) treated d. Control (Untreated)

Figure 1B. Efficacy of synthetic elicitors against *Fusarium oxysporum* f. sp. *cicieris* under glasshouse conditions in cv. JAKI 9218

II. Dynamic changes in defence enzymes activities

(i). Peroxidase (PO): Its activity increased significantly up to 18 days in all treatments, and thereafter declined. The maximum peroxidase activity was observed in the conc. of (3 mM) of salicylic acid (1.59) followed by benzothiadiazole [Bion] (1.50) treated plants. The least level of peroxidase was recorded in healthy (untreated) plants of (0.70) changes in absorbance min/g of fresh tissue (Figure 2a). These results revealed that peroxidise activity increased up to 4 days after inoculation challenged with *Fusarium oxysporum* f. sp. *ciceris* and *M. phaseolina* in chickpea (30) and combined application of benzothiadiazole [Bion] + humic acid (0.25 g/lit + 4 g/lit) on soybean cv. Giza 111 (17,15).



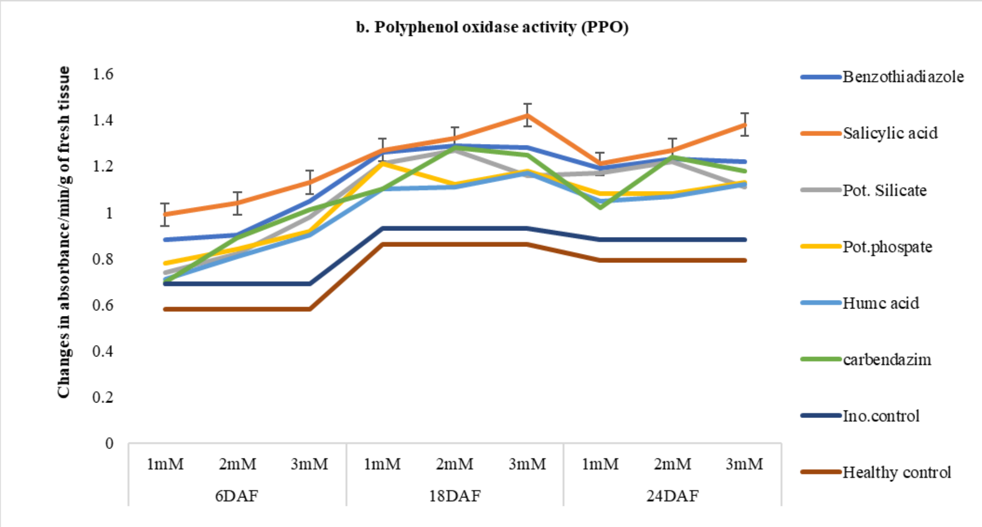


Figure 2. Dynamic changes of peroxidase (a) and polyphenol oxidase (b) activities against *F. oxysporum* f. sp. in chickpea by synthetic elicitors

(ii). Polyphenol oxidase (PPO): Its activity also increased significantly up to 18 days in all treatments and thereafter declined. The maximum polyphenol oxidase activity was observed in the conc. of (3 mM) salicylic acid (1.42), followed by benzothiadiazole [Bion] (1.28) treated plants. The healthy control recorded lesser polyphenol oxidase activity (0.86) changes in absorbance min/g of fresh tissue (Figure 2b). It was reported that arbuscular mycorrhizae indirectly induced the polyphenol oxidase (PPO) level in leguminosae crops (common bean) by nodulation and induced the salicylic acid derived from soil rhizobiome regions (45).

(iii). Superoxide dismutase (SOD): Its activity also significantly increased up to 18 days in all the treatments and thereafter declined. The maximum superoxide dismutase activity was observed in the conc. of (3 mM) salicylic acid (7.60), followed by benzothiadiazole [Bion] (6.80) treated plants. The healthy control recorded lesser super oxide dismutase activity of (3.25) Unit / min / g of fresh tissue (Figure 3c). These reports revealed that chickpea plants treated with 1.5 mM conc. of salicylic acid rapidly express the several isoforms of superoxide-dismutase against *Fusarium oxysporum* f. sp. *ciceris* (29).

Total phenols

The salicylic acid (3 mM conc.) recorded the higher amount of total phenol content 19.24 (µg of catechol/min/g of fresh tissue) up to 18 days and thereafter declined. This was followed by benzothiadiazole [Bion] (17.15), and the healthy control recorded lesser accumulation of phenol content 3.33 in chickpea cv. JAKI 9218 (Figure 3d). Above results were confirmed by benzothiadiazole [Bion] + humic acid treated soybean *cv.* Giza111 which expressed highest accumulation of phenolic level during combined application (17,15). Bioactive compound thiamine (or) different copper sources treated pear plants had high accumulation of phenolics against *Alternaria alternata* in citrus (37,18).

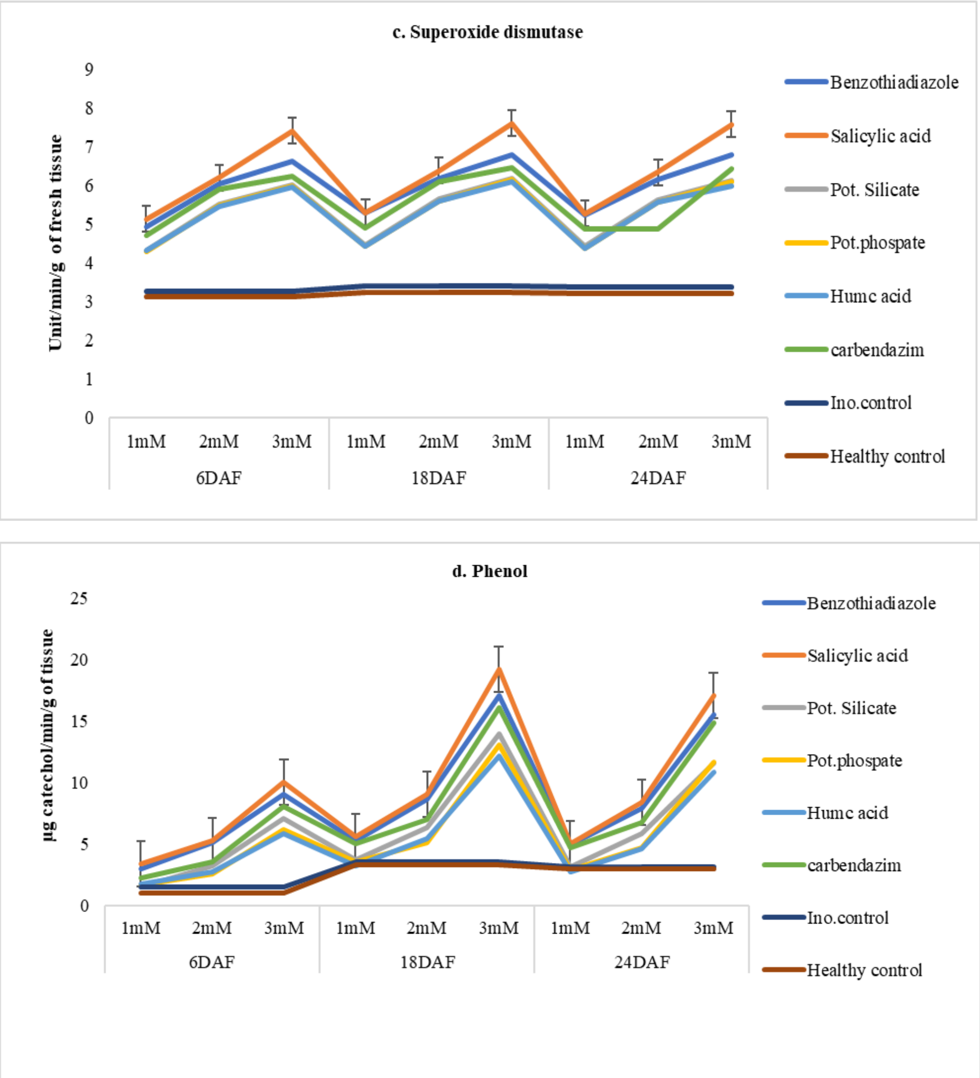
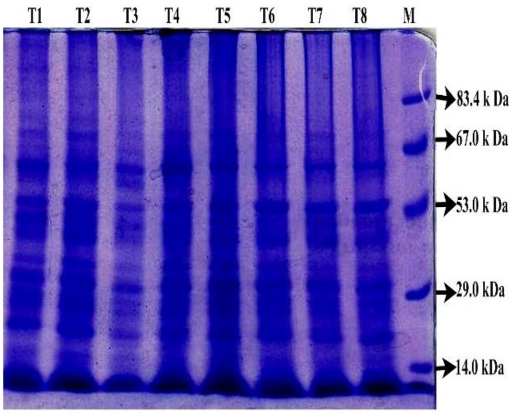


Figure 3. Dynamic changes of superoxide dismutase (c) and phenol (d) activities against to *F. oxysporum* f. sp. *ciceris* in chickpea by synthetic elicitors

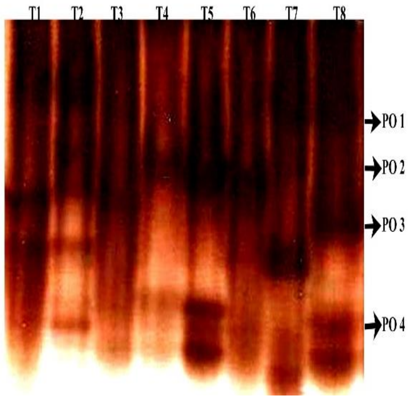
III. Quantification of defence enzymes

Crude protein analysis - SDS PAGE

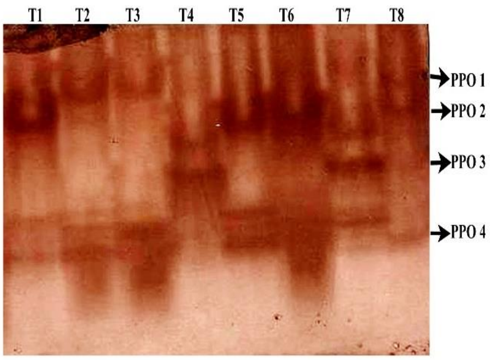
The synthetic chemical elicitors treated plants expressed 5 proteins with molecular weight *viz*., 14, 29, 53, 67and 83.4 kDa. However, in healthy plants, only 4 proteins with molecular weight of *viz*., 14, 29, 53 and 67 kDa were observed. In untreated inoculated plants only three proteins with molecular weight, of *viz*., 14, 29 and 53 kDa were observed (Figure 4a). These results are in agreement with several findings such as (34) which reported that 1.0 mM salicylic treated chickpea plants exhibited 10-200 kDa proteins compared to uninoculated chickpea plants within 24hrs after application against *Ascochyta rabiei*. Riboflavin treated chickpea plants exhibited 20-97 kDa protein in SDS-PAGE analysis against with wilt and root rot pathogens (30), and additionally, hexanoic acid treated citrus plants expressed PR-2 proteins against *Xanthomonas campestris* pv. *citri* and *Alternaria alternata* (12,14).



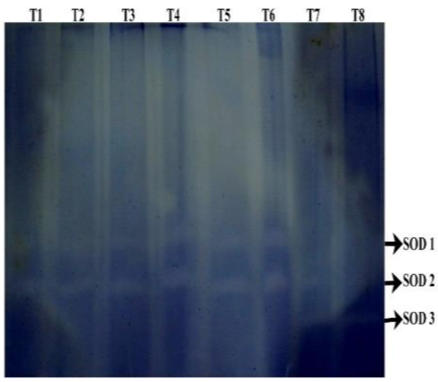
a. SDS-PAGE analysis for crude proteins



b. Expression of peroxidase



c. Expression of polyphenol oxidase



d. Expression of superoxide dismutase

Figure 4. Quantification profiling of crude protein (a) and defence enzymes isoforms (b,c&d) on chemo-fortified chickpea *cv*. JAKI 9218 under *in vitro*

Treatment details (Conc. of 3 mM) by chemo-fortification on seeds (*cv*. JAKI 9218)

T1: Benzothiadiazole (Bion), T2: Salicylic acid, T3: Potassium silicate, T4: Potassium PO_4, T5: Humic acid, T6: Carbendazim (fungicide - check), T7: Inoculated control, T8: Uninoculated control

(i). Peroxidase (PO): The treated chickpea plants were used for analysis for native PAGE of peroxidase (PO). The 3 mM conc. of salicylic acid treated plants showed four isoforms, *viz*., PO1, PO2, PO3 and PO4 and the other treatments (Figure 4b). These results are in agreement with (16) methyl jasmonate (MeJA) and benzothiadiazole [Bion] co-inoculated in melon plants exhibited peroxidase isoforms from pI 3.8 to 9.3. against to *Monosporaascus cannonballus*. Molecular weight of 43 kDa peroxidase from riboflavin chickpea plants challenged with wilt and root rot pathogens under *in vitro* (2).

(ii). Polyphenol oxidase (PPO): Elicitor treated plants with 3 mM conc. of salicylic acid showed four isoforms, *viz*., PPO1, PPO2, PPO3 and PPO4 and the other treatments, *viz*., healthy and inoculated control plants showed one isoform of PPO4 with less intensity (Figure 4c). These results are in agreement with Rashid *et al.* (29) who reported that increasing the conc. of salicylic acid exhibited more isoforms in chickpea plants from 1-2 mM against pathogen stress. Induction of several isoforms, *viz*., PPO1, PPO2, PPO3 and PPO4 were expressed due to the application of liquid formulation of Pf 1 in tomato plants (3). Combined application of EPCO16, EPC5 and Pf 1 in chilli plants expressed three isoforms, *viz*., PPO1, PPO2 and PPO3 (38). defence activity of enzyme PPO was increased by the application of biocontrol agents, plant products and challenge inoculated with *Alternaria alternata* infecting chrysanthemum (3).

(iii). Superoxide dismutase (SOD): In this study, 3 mM conc. of salicylic acid treated plants showed four isoforms, *viz*., SOD1, SOD2 and SOD3 and the other treatments (Figure 4d). These results are in conformity with Nazari *et al.* (24) the changes of SOD activity were apparent by 72 h after bacteriocin antibiotic from *Bacillus* strains treatment of soybean leaves. Tomato plants treated with combined PGPR strains are triggered the total peroxidase and superoxide dismutase (SOD) activities were increased to a greater extent than those in the control treatment (1).

CONCLUSIONS

We did this study on a virulent wilt pathogen (*F. oxysporum* f. sp. *ciceris*) in chickpea using an alternative approach of using synthetic triggers in a controlled environment. In this study, toxic wilting was managed by chemically fortifying six different synthetic elicitors (benzothiadiazole, salicylic acid, potassium silicate, potassium phosphate, humic acid, and carbendazim) at three different concentrations (1.2 and 3 mM) used against virulent pathogen of wilt in cv. JAKI 9218. Among them, salicylic acid showed a rapid form of protective enzymes and proteins against *F. oxysporum* f. sp. *ciceris* reduces the incidence of wilt to the greatest extent from the preliminary to vegetative stages of plant growth. These discoveries provide new approaches to future disease management that are environmentally friendly and affordable, while at least using concentrated small molecule compounds to achieve better results in reliably reducing disease incidence. It will generate an outstanding approach in future crop protection.

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DECLARATION

Kanchaveli, Sh. S. designed and planned experiment. Chkhubadze G. Conducted experiment in greenhouse and plantation. Shainidze O. T. conducted experiments in the laboratory and wrote article.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct.

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