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ORIGINAL ARTICLE

Phytochemical and Antioxidant Profiling of *Allium Sativum* Germinated under Biotic and Abiotic Stress

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ABSTRACT

Purpose: The purpose of the study is to evaluate the potential of *Allium sativum* under biotic and abiotic stress in the quest to obtain more efficient phytoconstituents with improved antioxidant properties.

Method: We investigated the effect of stress induction through biotic (*Fusarium solani*) and abiotic (NaCl salt of two different concentrations i.e. 50 mM and 100 mM) means in *Allium sativum* (garlic) to uncover its phytochemical and antioxidant potential. After stress induction, the leaves were harvested at different day's post-inoculation (dpi) and analyzed for phytoconstituents and antioxidant assays.

Results: The data showed statistically significant differences among proteins, reducing sugars, total soluble sugars, proteases and amylase of the samples at different dpi under biotic and abiotic stress ($P<0.05$). Moreover, total flavonoid, phenolic constituents, DPPH, ascorbic acid, catalase, peroxidase and superoxide dismutase were significantly increased under stress ($P<0.05$). The phytochemical and antioxidant activities were altered that proved its effectiveness under induced conditions.

Conclusion and Practical Implications: The results obtained indicated that the *Allium sativum* (garlic) grown under biotic and abiotic stress have certain phytoconstituents with improved antioxidant activity that may serve as a rich source for antioxidants and proteins that may be used as an alternate to synthetic formulations in food and pharmaceuticalsto improve health outcome.

Keywords: *Allium sativum*, antioxidant, biotic, abiotic, phytoconstituents, stress

INTRODUCTION

Since long, plants have been used in pharmaceutical industry and provide a natural source of potential biochemical and antioxidant compounds. Several studies have been conducted to explore medicinal value of traditional plants and some of the compounds from natural products have been approved as drugs. Rendering WHO reports, 80% population throughout the world predominantly from developing states are focusing towards medicine derived from plants due to the side effects accompanied with synthetic drugs[(1)]. Plants can easily espouse the mechanism of biomolecules synthesis and production under induced conditions. One of the main mechanism is dependent upon the influence of ROS (reactive oxygen species) including hydrogen peroxide, superoxide radical and most reactive hydroxyl radical that are altered under stress condition both biotic and abiotic[(2)]. The activities of antioxidant synthesizing enzymes also demonstrate the ROS detoxification mechanism in plants. These enzymes include SOD (superoxide dismutase), POD (peroxidase) and CAT (catalase) catalyzing hydrogen peroxide scavenging with the formation of oxygen and water[(3)]. A recent study reported the effect of heat on antioxidant activities of *Astringent Persimmon* fruit[(4)]. Another study highlighted the significance of geographical variation in physical, biochemical and antioxidant activities of *Vaccinium myrtiluus*[(5)].

Biotic and abiotic stress has been reported to induce biological activities of plants and the antioxidant activities of their compounds are enhanced under this stress. The production of xanthone in *Hypericum perforatum* caused increased antioxidant properties under biotic stress[(6)]. The volatile compounds of *Abelmoschus esculentus* (okra) are enhanced by biotic stress[(7)].

However, the phytochemical analysis and antioxidant profiling of *Allium sativum* (garlic) under biotic and abiotic stress has not been performed so far as per review literature. Due to increasing rate of maladies particularly cancer, there is an emergent need to find out more efficient phytoconstituents and antioxidant compounds that may have the potential to fight against the reactive oxygen species (ROS) as ROS directly influence the gene expression and ultimately control cell growth, cell cycle and apoptosis. The present study was designed to evaluate the phytochemical and antioxidant potential of *Allium sativum* (garlic) under biotic and abiotic stress in the quest for improvement of natural products with enhanced capacity to produce biochemical and antioxidant compounds with potential health benefits.

MATERIALS AND METHODS

Germination: The *Allium sativum* (garlic) bulbs were surface sterilized by using distilled water and air dried until moisture evaporated. The bulbs were then germinated in control conditions in botanical garden, Department of Botany, University of Agriculture Faisalabad.

Biotic induction: For biotic stress, *Fusarium solani (F. solani)* was inoculated deep inside the young seedling roots of *Allium sativum* (garlic). Then, the biotic stress induced plants were harvested after specific time designated as day post-inoculation (dpi) from dpi 1 – 7. On each day, a control sample without fungal induction and salt stress was also harvested and collected.

Abiotic induction: Abiotic stress was given by salt induction of 50 mM and 100 mM NaCl solutions to the young seedlings. The inoculated plants were harvested at different day's post-inoculation (dpi) from dpi 1 – 7. On each day, a control sample without fungal induction and salt stress was also harvested and collected.

Extraction of plant samples: The bulbs of *Allium sativum* were further processed for extraction of plant samples using phosphate buffer saline of pH 7.8 (10mM K_2HPO_4, 100mM KH_2PO_4, 100mM KCl, 2mM EDTA) as per protocols in accordance with the literature[(8,9)]. Briefly, the dirt free samples were prepared by serial washing using tap and distilled water and then kept moisture free. Further, the uniform size pieces of plants were made and mixed with phosphate buffer saline (PBS) with 1:2 ration for extraction. Then the mixture was undergone fine grinding procedure with PBS by keeping at 4°C. The next step was to centrifuge (Centrifuge H-200 NR; Kokusan, Japan) the mixtures at 10,000 rpm for 10 min at 4°C. The residues remaining in the pellet were discarded and the supernatant first separated then filtered to remove large particles. Thereafter, the filtrates were saved at 4°C till future processing.

Biochemical analysis: Biochemical analysis of the biotic and abiotic stress induced *Allium sativum* was determined using commercially available kits. Biochemical parameters analyzed were total protein content, reducing sugars, total soluble sugars, protease and amylase. Total protein content was determined using Bradford method[(10)]. The reducing sugars and TSS (total soluble sugars) were tested by a modified method of Sadasivam and Manickam[(11)]. Protease and amylase assay were performed using

Casein digestion protocol provided by Drapeau *et al*.[12] and Varavinit *et al*.[13] with some modifications.

Phytochemical investigation: Phytochemical investigation includes TFC (total flavonoid content) and TPC (total phenolic content). TFC and TPC were analyzed by FC reagent (Folin-Ciocalteu reagent) and Vaniline reagent method, respectively, as described by Bozin *et al*.[14].

Antioxidant profiling: Antioxidant profiling include ascorbic acid, SOD (superoxide dismutase), CAT (catalase), POD (peroxidase) and DPPH radical scavenging. The 2,6-dichloroindophenol (DCIP) method was used to measure ascorbic acid in *Allium sativum* extracts as described by Kevers *et al*.[15]. The SOD activity was checked by its capacity to inhibit the NBT (nitroblue tetrazolium) photoreduction method of Stajner and Popovic[16]. Catalase and peroxidase activities were measured using modified method of Liu *et al.*[17]. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was determined as described by Bozin *et al*.[14].

Statistical analysis: The data measured from all the parameters was analyzed statistically using IBM SPSS Statistics (V21 for Windows). The experiments were performed in triplicate and the mean±Standard deviation of biochemical and antioxidant assays of induced (biotic and biotic stress) and control (not induced) group samples of *Allium sativum* was calculated. The average readings were represented with graphical demonstration. The comparison of means between groups was analysed with one way ANOVA (analysis of variance) and *P*-value <0.05 with statistically significant difference was considered.

RESULTS

Germination and induction: In the present study, *Allium sativum* was successfully germinated in the sand pots. Fungal induction by *Fusarium solanii* and salt stress of different concentrations (50 mM and 100 mM salt) was used to explore the potential of *Allium sativum* under biotic and abiotic stress, respectively. The samples were collected for 7 consecutive days of post-inoculation (dpi) and stored for further biochemical and antioxidant profiling.

Extraction of *Allium sativum* samples: The extraction of samples from the *Allium sativum* plant was obtained with 50 – 70% yield with phosphate buffer saline (PBS; pH 7.8).

Biochemical analysis: Different biochemical parameters were quantified in the extracts of *Allium sativum* to find out the inherent potential of the plant under stress conditions induced by *Fusarium solani* and 50 mM and 100 mM of NaCl. The results of different phytometabolically important biochemical parameters like proteins, reducing sugars, total soluble sugars, proteases and amylase were represented (Figure 2). The data showed statistically significant differences among protein content of the samples at different dpi under biotic and abiotic stress (*P*<0.05).The maximum protein content (124.24±0.03) was obtained under fungal induction at dpi 7 followed by control group, 50 and 100 mM salt induction samples respectively. Minimum protein content (48.20±0.07) was obtained in 100 mM salt induction (Figure 1).

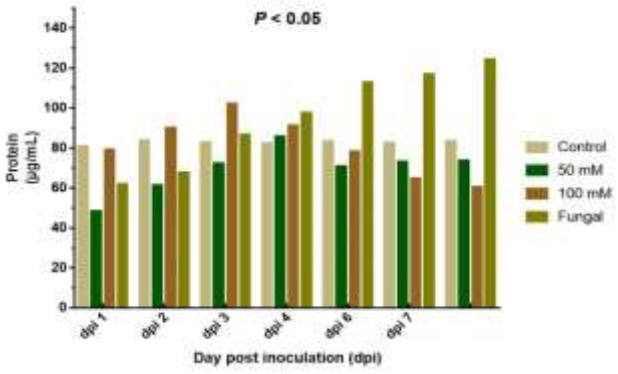

Figure 1: Total protein content determination of *Allium sativum* extracts under different concentrations of salt i.e., 50 Mm, 100 Mm and fungal stress compared with control at different dpi (*P*<0.05).

The highest reducing sugar content was recorded in dpi 7 and lowest in dpi 2 as shown (Figure 2a). The study showed highest concentration of reducing sugars at dpi 6 in 50 mM salt stresses induced followed by 100 mM salt induction, control group, and fungal induction; respectively as shown (Figure 2a). The total soluble sugar content was also significantly different among all treatment groups and control groups (*P*<0.05). The lowest concentration was observed at dpi 1 in fungal-induced samples for reducing sugars, total soluble sugars, protease and amylase (Figure 2a-d).

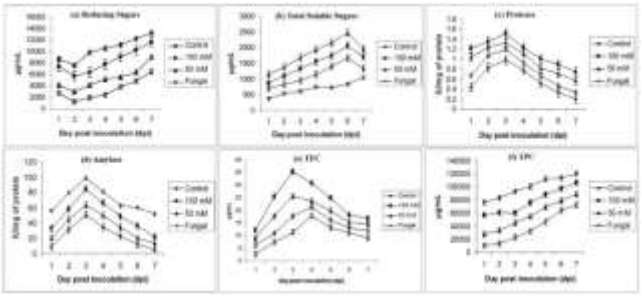

Figure 2: Phytochemical analysis of biotic and abiotic stress induced *Allium sativum* (garlic) extract (a) Reducing sugars in *Allium sativum* seedling extracts at different days post-inoculation germinated under different biotic and abiotic stress (b) Total soluble sugars in *Allium sativum* extracts germinated under fungal and salt stress at different day post-inoculation (c) Comparison of protease in extracts of *Allium sativum* of fungal and salt stress samples collected at different day post-inoculation (d) Comparison of amylase in extracts of *Allium sativum* of fungal and salt stress samples collected at different day post-inoculation. (e) Comparison of TFC in *Allium sativum* at different day post-inoculation under fungal and salt stress inductions (f) Comparison of TPC in seedling extracts of *Allium sativum* of fungal and salt stress samples collected at different day post-inoculation.

Determination of Phenolic constituents

Total Flavonoid content (TFC): The TFC assay of *Allium sativum* was observed to possess the highest concentrations at dpi 3 in 100 mM salt induction followed by 50 mM salt induction at dpi 3, control group at dpi 4, and fungal induction at dpi 4; respectively. While the lowest concentration was observed in dpi 1 samples as shown in (Figure 2e). There was a significant difference in TFC content among different salt concentrations and fungal stress at different dpi (P<0.05) (Figure 2e).

Total phenolic content (TPC): A quite good profile of TPC was recorded in *Allium sativum* as shown (Figure 2f). Highest TPC was observed at dpi 7 in the control group followed by 100 mM, 50 mM, and fungal induction respectively. The lowest concentration was observed in dpi one.

Antioxidant assays

DPPH radical scavenging assay: In the DPPH assay, the ability of induced garlic extracts to act as donors of hydrogen atoms or electrons in the conversion of DPPH radical into its reduced form was evaluated. The highest concentration was observed at dpi 4 in 100 mM salt induction followed by 50 mM salt induction and fungal induction; respectively. The lowest concentration was observed in the control group as presented (Figure 3a).

Ascorbic acid: Results illustrated in the current study indicated that the highest ascorbic acid concentration was found in the extracts of *Allium sativum* at dpi 3 in 100 mM salt inductions followed by 50 mM salt induction at dpi 7 (Figure 3b).

Superoxide dismutase (SOD): The highest concentration was observed at dpi 3 in 100 mM salt-induced stress followed by 50 mM salt induction, control group, and fungal induction; respectively. The lowest concentration was observed in fungal-induced samples as shown (Figure 3c).

Catalase activity: The highest catalase activity was observed in dpi 3 samples in the control group followed by 100 mM salt concentration, 50 mM salt concentration, and fungal induction; respectively (Figure 3d).

Peroxidase activity: The comparison of the peroxidase (POD) specific activity in extracts of *Allium sativum* was presented (Figure 3e). The highest specific activity of peroxidases (POD) was recorded in 100 mM salt induction at dpi 4 followed by 50 mM salt induction, control group, and fungal inductions respectively.

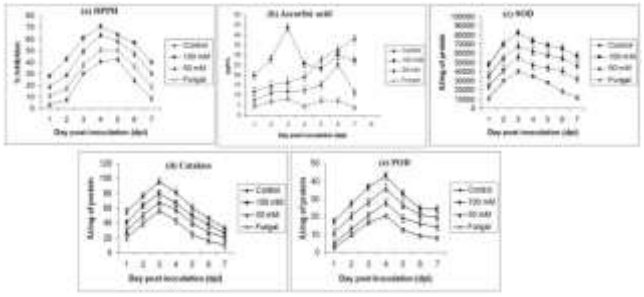

Figure 3: Antioxidant profiling of *Allium sativum* under biotic and abiotic stress. (a) Comparison of DPPH in *Allium sativum* at different day post-inoculation under fungal and salt stress inductions (b) Comparison of Ascorbic acid in *Allium sativum* at different day post-inoculation under fungal and salt stress inductions (c) Specific activity of SOD in *Allium sativum* extracts at different day post-inoculation under fungal and salt inductions (d) Comparison of catalase in extracts of *Allium sativum* of fungal and salt stress samples collected at different day post-inoculation (e) Comparison of peroxidase in extracts of *Allium sativum* of fungal and salt stress samples collected at different day post-inoculation.

DISCUSSION

The study represented the effect of biotic and abiotic stress on the phytochemical, biochemical, and antioxidant profiling of *Allium sativum* (garlic) that is previously reported to contain bioactive components having antioxidant potential. As studies suggested that biotic and abiotic stress can induce phytoconstituent and may help to increase antioxidant activity of certain medicinal planst. The present research was designed to evaluate its phytochemical and antioxidant potential of garlic under stress conditions. The garlic was germinated under different salt concentrations and fungal stress and significant changes were observed. The maximum protein content of *Allium sativum* was obtained under fungal induction (Figure 1). The higher concentration of proteins may be due to biochemical changes that have been induced as a result of fungal stress. The increasing trend of reducing sugar and total soluble sugars from lower concentration at dpi 1 to higher at dpi 7 and dpi 6, respectively showed the positive effect of biotic and abiotic induction (Figure 2a-b). However, the enzymatic assays i.e., protease and amylase showed highest activity at dpi 3 (Figure 2c-d). Previously, studies have been conducted to investigate the comparative activity of antioxidant compounds present in plant extracts that showed various effects according to the model used for assessment[14]. A recent study reported enzymatic and functional activity of allium species and their phytochemical, antioxidant profiling[18].

Different flavonoid content observed in the salt and fungal induced garlic extracts showed modification in garlic extracts that is evident from the results obtained (Figure 2e). These variations may be due to altered mechanism of action under stress conditions. Total phenolic profile showed increasing concentration with day post inoculation (Figure 2f). The maximum phenolic content was observed at dpi 7 in the control group followed by 100 mM, 50 mM, and fungal induced group, respectively. A study reported that immature garlic plant have higher content of quercetin equivalents i.e., 6.99 lg QE/g than mature garlic bulbs (5.78 and 4.16 lg QE/g) (14). Total phenolic content is considered good index to measure antioxidant potential of compounds. Oxidative stress due to imbalance production of free radicals and endogenous mechanism of antioxidant defense in different cells and tissues plays an important part in the start and as well as progression of nearly all these situations. The antioxidant properties of *Allium sativum* and its various preparations are well known in literature[14,19,20] but its antioxidant effect under biotic and abiotic induction investigated in the present research has not been conducted previously. The reduced levels of TPC are possibly due to presence of more sulfur containing constituents and terpenoids induced by salt and fungal stress. In the DPPH assay, the ability of induced garlic extracts to act as donors of hydrogen atoms or electrons in the conversion of DPPH radical into its reduced form was evaluated. The highest concentration was observed at dpi 4 in 100 mM salt induction followed by 50 mM salt induction and fungal induction; respectively. The lowest concentration was observed in the control group as presented (Figure 3a). Researchers reported the DPPH assay for garlic extract described that garlic extracts can reduce DPPH (purple-colored stable radical), into the DPPH-H (yellow-colored), reaching 50% reduction[14]. Results illustrated in the current study indicated that the highest ascorbic acid concentration was found in the extracts of *Allium sativum* at dpi 3 in 100 mM salt inductions followed by 50 mM salt induction at dpi 7 (Figure 3b). The lowest concentration was observed at dpi 1 both in the control and induced groups, respectively. The ascorbic acid levels in most of the vegetables and fruits was found fairly constant during storage. However, some studies reported that the ascorbic acid reduced rapidly during the storage conditions[15]. A recent study reported that ascorbic acid could itself can increase the secondary metabolites and antioxidant potential of commonly used bean under abiotic stress[21]. Superoxide dismutase (SOD) showed higher concentration at dpi 3 in 100 mM salt-induced stress followed by 50 mM salt induction, control group, and fungal induction; respectively (Figure 3c). SOD catalyzes the destruction of the O^{2-} free radical. It protects oxygen-metabolizing cells against the harmful effects of superoxide free-radicals. SOD protects hyaluronate against depolymerization by free radicals and indicated that exogenous SOD might have an anti-inflammatory effect. The SOD activity was measured using its capability to hinder the nitroblue tetrazolium (NBT) photoreduction[16]. The literature highlighted that salt stress has increased the activities of leaf mitochondrial Mn-SOD and chloroplastic Cu/Zn SOD, which is considered the primary scavenger in the detoxification of active oxygen species in plants and converts superoxide to oxide, and offers to protect cells against super oxide-induced oxidative stress[16]. Our results supported the view that SOD activity increased concurrently with increasing salinity. The ability of the fast, effective antioxidant response in stress situations can improve tolerance. The processes connected with the enhancement of ROS could induce the protective mechanisms in plants[22,23]. Catalase is a ubiquitous antioxidant enzyme that is present in most aerobic cells. It is involved in the detoxification of hydrogen peroxide (H_2O_2), which is a toxic product of normal aerobic metabolism and pathogenic reactive oxygen species (ROS) production. In this enzyme assay, the peroxidation function of catalase was utilized for the determination of enzyme activity of plant extracts[24]. The maximum level of catalase activity was observed in dpi 3 samples in the control group followed by 100 mM salt concentration, 50 mM salt concentration, and fungal induction; respectively (Figure 3d). The study indicated that the enzyme activity was reduced under induced conditions with salt-induced groups having higher values than the fungal-induced group. Literature reported that abiotic-induced stress shows a decrease in catalase (CAT) activity[25]. Recent study reported catalase activity of herbal plants including *N. hindustana*, *A. albiflora* and *V. negundo* against multiple sclerosis[26]. Our results also showed a decrease in catalase activity, which led to the accumulation of toxic levels of hydrogen peroxide. Peroxidases (POD) showed higher value in 100 mM salt induction at dpi 4 followed by 50 mM salt induction, control group, and fungal inductions respectively (Figure 4e). This indicated that POD activity increased with salt stress and the same trend was reported previously that salt stress enhanced the activity of peroxidase (POD)[25]. Peroxidase (POD) is an important enzyme, able to scavenge H_2O_2, which is a major substance degraded by SOD[17]. It also protects the membrane exposed to oxidation stress[27]. Peroxidases are among the biomarkers of the antioxidant system in plants under different physiological stresses. The variety of response has been

investigated when measuring POD levels of induced soybean[28]. Collectively, the study showed altered activity of phytoconstituents and antioxidants compounds found as a result of germinating *Allium sativum* i.e., garlic under salt and *Fusarium solanii* stress. The induction through biotic and abiotic stress may be used to enhance the bioactive components present in the garlic that ultimately may increase effectiveness of the compounds found in garlic for use in food, nutraceuticals and pharmaceutical applications.

CONCLUSION

The present research work was chalked out to screen biochemical and antioxidant potential of *Allium sativum* (garlic) grown under biotic and abiotic stress. Bulbs of *Allium sativum* were induced with biotic (*F. solani*) and abiotic (NaCl salt of two different concentrations i.e. 50 mM and 100 mM) stress. Higher protein content was observed in 50 mM salt-induced extracts. The results gathered from experimental studies reflected that biotic and abiotic stress may play a key role to enhance activity of biochemical compounds, enzymes, phytoconstituents and antioxidants present in *Allium sativum* (garlic). The improved biochemical, phytochemical and antioxidant potential under induced conditions may be used in pharmaceuticals, food and nutraceutical industry.

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