The Journal of Medicinal Plants and By-Products

Original Article

Antioxidant and Antimicrobial Properties of Garlic as Affected by Nitrogen and Selenium Concentrations

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Article History: Received: 20 December 2023/Accepted in revised form: 07 June 2024

Highlights

- The positive effect of nitrogen and selenium on the antimicrobial and antioxidant properties of garlic
- Increasing the antioxidant properties and allicin of garlic
- For the first time nitrogen and selenium in garlic has been investigated.

ABSTRACT

Antibiotic resistance has contrived the use of medicinal plants with fewer side effects instead of common drugs. Garlic has sulfur-containing organic compounds and has broad antimicrobial properties against bacteria, even in the lowest concentrations. Allicin or diallyl disulfide is the main garlic sulfur compound with antimicrobial activity. This research investigated the different impacts of nitrogen (N) and selenium (Se) concentrations on garlic clove antioxidant and antibacterial activity. Three concentrations of Se, such as 0, 5, and 10 mg L^{-1} sodium selenate and N, containing 0, 50, 100, and 150 kg ha^{-1}, were examined on garlic's antioxidant and antimicrobial properties**.** The results showed that the antioxidant activity (66.77%) improved in four nitrogen concentrations. Selenium decreased the allicin content of garlic clove. The highest inhibition of the growth rate was observed in *Escherichia coli* at 12.00 mm, *Pseudomonas aeruginosa* at 1.73 mm, *Bacillus subtilis* at 8.95 mm, and *Staphylococcus aureus* at 10.90 mm were obtained in 150 kg ha^{-1}N coupled with 10 mg L^{-1} sodium selenate. The lowest inhibitor of the growth of all four bacteria was observed in the control treatment. According to the results, an increase in antioxidant activity was associated with an increase in the antimicrobial properties of garlic. The use of selenium and nitrogen increased the inhibitory influence of bacterial growth.

Keywords: Antioxidant capacity, Ascorbic acid, Sodium selenate

INTRODUCTION

Garlic (*Allium sativum* L.) is a plant belonging to the Alliaceae family that is used as a spice and seasoning, as well as a medicinal plant in the treatment of various disease types [1]. Garlic has a high anticancer effect, which is related to its sulfur compounds [2]. It has useful effects on the cardiovascular system and human immunity [3 and 4]. Alliums with healthy tissue mainly contain cysteine sulfoxide. When the tissue is crushed, the allinase enzyme in the vacuole is released and converts cysteine sulfoxides into thiosulfinates. Which, in addition to nutritional effects, have antibacterial and antifungal effects against all types of Gram-positive and Gram-negative bacteria [5]. It has been reported that the antibiotic properties of one mg of allicin are equivalent to 15 IU of penicillin. [6]. The sulfoxides amount typically depends on genetic factors and additional storage conditions after garlic is harvested. The antimicrobial activity of garlic is higher than that of onion. For example, 7 contains 1-4% garlic completely prevents the growth of *Escherichia coli* and *Staphylococcus aureus*, while 4% onion extract prevents the blossoming of strains [7]. Wook Kim *et al*. (2004) showed that garlic 25 and 100 mg L^{-1} has an antimicrobial effect on *Candida utilis* and *Staphylococcus aureus* strains [8].

Selenium (Se) is an essential element, showing powerful antioxidant properties [9]. Unfortunately, the uneven distribution of selenium over the Earth's surface causes broad Se-deficient areas. The biologically active form of selenium is selenoproteins, and in mammals, selenium binds to proteins as a cofactor [10 and 11]. The similarity in chemical properties between selenium and sulfur triggers the processes of sulfur replacement by selenium in biological systems, resulting in the formation of Se-containing amino acids, proteins, carbohydrates, and other biologically active compounds [12]. By inhibiting the oxidation of low-density lipoproteins (LDL cholesterol), selenium reduces the risk of cardiovascular diseases [13]. Selenium compounds induce apoptosis (cell death) in cancer cells and stimulate the immune system [14 and 15].

Research is uncovering that the availability of plant nutrients can be a significant factor in determining secondary metabolism and antioxidant within plants [16]. Nitrogen (N) plays a role in the structure of biological molecules such as proteins, enzymes, coenzymes, nucleic acids, and cytochromes. Nitrogen is one of the original growth factors in controlling yield and quality of plants [17]. In the yarrow plant (*Achillea millefolium* L.), 75 kg h^{-1} of urea application increased the antibacterial and antiviral properties [18]. While nitrogen is an essential nutrient element for crop growth and quality, there is little information on the nitrogen supply impact on the antioxidant activity of *Allium*.

Plants have antimicrobial effects and inhibit the growth of bacteria. So far, the nitrogen and selenium influence on the antimicrobial properties of plants, including garlic, has not been studied. Therefore, in this research, for the first time, the different effect concentrations of nitrogen and selenium on the antimicrobial properties of the alcoholic extract of garlic on the growth rate of 4 gram-positive and gram-negative bacteria was investigated.

MATERIAL AND METHODS

This study was conducted in the experimental field of Razi University (Kermanshah 34.377º N, 47.0078º E), in 2022. This research was carried out as a factorial experiment based on a randomized complete block design with two factors of different concentrations of nitrogen and selenium in three replications of landrace garlic of Kermanshah. The first factor included 4 concentrations of nitrogen containing 0, 50, 100, and 150 kg ha^{-1}, and the second factor included three concentrations of selenium, such as 0, 5, and 10 mg L^{-1} of sodium selenate. Nitrogen fertilizer from the source of urea (46% N) was added to the soil in two stages, one at the same time as planting (first half of November) and the other during the bulbing stage (May). For each treatment, was added the fertilizer to each plot at the designated times, and irrigation was performed immediately. Selenium foliar spraying in the form of sodium selenate salt was done manually in the evening and at the same time with nitrogen top-dressing fertilizer. After preparing the land, plots (3×2 m^2) were considered. Cloves were planted in rows at a depth of 5 cm in early November of 2022. The distance between planting rows was 30 cm, and the distance between plants on the row was 10 cm. Immediately after the planting of the cloves, leakage irrigation was carried out, so that the thriving of the roots in the cloves was stimulated, and they were well established in the soil in the subsequent irrigations according to the custom of the region, the weather, soil conditions, the amount of rainfall, and environment temperature.

Harvesting was done when ripening indications were observed in the plants and complete drying of aerial organs. Sampling was carried out to measure the traits of interest after removing the marginal effects from the surface of 0.4 m^2.

Antioxidants

Ascorbic Acid (AA)

It was defined by visual titration of plant extracts in 6% trichloracetic acid with Tillman's reagent [19]. Three grams of fresh garlic cloves homogenates were ground in a porcelain mortar with 5 mL of 6% trichloracetic acid and quantitatively transferred to a measuring cylinder. The volume was brought to 60 mL using trichloracetic acid, and the mixture was filtered through filter paper 15 min later. The concentration of ascorbic acid was determined from the amount of Tillman's reagent that went into the titration of the sample.

Antioxidant Activity (AOA)

The antioxidant capacity of garlic cloves was measured by the DPPH method. In this method, the neutralizing activity of 2-2 diphenyl-1-picrylhydrazyl radicals was distinctived by the methanolic extract by spectrophotometric method at a wavelength of 515 nm, which follows Lambert's law, and the reduction of its absorption has a linear relationship with the amount of antioxidant. The more the antioxidant substance is added, the more DPPH is consumed, and the purple color tends to yellow. DPPH is a purple compound that becomes easily a radical due to the presence of phenyl groups in its structure and is a source of free radicals. This compound changes color from purple to yellow by taking an electron from the antioxidant compound [20].

DPPH radical neutralization activity was calculated based on the following formula.

DPPH radical neutralization percentage formula:

In this formula, AC: Absorbed DPPH radical without any antioxidant as a control

As: Absorbed DPPH with the extract. Methanol was used as a blank.

Allicin

Allicin was analyzed with a slightly modified method by Liang et al. (2013) [21]. The bulbs were peeled, and the cloves were roughly sliced in a small blender. Then, 20 g of material (5 g per tube) and 25 ml of double distilled water, were added to 4 centrifuge tubes. The mixture was homogenized using a T-25 Ultra-Turrax for 1 min at 11.000 rpm. After homogenization, the samples for allicin determination were transferred onto a shaker at room temperature for 30 min, centrifuged at 10.000 × g for 15 min, and filtered through a 0.45-μm polyamide filter Chromafil. The samples were then stored at −80 °C and subsequently lyophilized. Garlic extracts were analyzed using the Agilent 1260 Infinity HPLC system (HP model 1050 system), consisting of a binary pump (Agilent 1260 Infinity model G1312B), an autosampler, and a diode array detector. The Agilent 1260 Infinity HPLC system was controlled by Agilent HPLC 2D Chemstation SW software. HPLC analysis was carried out using a C18 column (Zorbax Eclipse Plus; 4.6 × 150 mm, 3.5 mm particle size; Agilent Technologies, Inc., Wilmington, DE, USA) and an analytical guard column (Agilent Eclipse XDB-C18; 4.6 ×12.5 mm, 5 mm particle size). The column was operated in isocratic mode, with a mobile phase of 0.08% formic acid in water: methanol (35:65, v/v) at a flow rate of 0.5 mL min^{-1}. The column temperature was maintained at 25 °C, and the samples at 4 °C. UV-Vis absorption spectra were recorded in the range from 200 to 400 nm, and allicin was noted at 220 nm. All the extracts were diluted in the mobile phase solution before the analysis and filtered through 0.45-μm PTFE syringe filters. Each standard solution and sample solution was analyzed in triplicate. Allicin standard was obtained from LGC (Middlesex, UK), and methanol for liquid chromatography LiChrosolv® was obtained from Merck. For detection of dry matter, 2 g of the frozen sample was freeze-dried for 22 h in a Gamma 2-20 lyophilizer (Christ, Germany), and the water content (%) was calculated from the difference between the masses before and after lyophilization.

Antimicrobial Activity

The Disk Diffusion test (DD) method, Padton Teb Company Product, 6 mm in diameter, was used to investigate the antibacterial effects of different concentrations of garlic cloves extract. To prepare the culture medium, 35 g of prepared Muller Hilton culture medium was dissolved in 1 L of distilled water and was autoclaved at 121 ºC and 1.5 ºA for 15 min. When was cooled in the environment under the biological hood distributed in sterile disposable Petri dishes. Four bacterial strains were used for this experiment (Table 1).

Table 1 Characteristics of bacterial strains used in this research

Row	The name of the bacterium	Gram +/-	Standard number
1	*Escherichia coli*	-	ATCC 25922
2	*Pseudomonas aeruginosa*	-	PTTC-1181
3	*Bacillus subtilis*	+	ATCC 12711
4	*Staphylococcus aureus*	+	PTTC-1189

To cultivate bacteria, single clones of the desired bacteria were transferred to Mueller Hilton agar culture medium and positioned in an incubator at 36 ºC for 24 h. A suspension with a concentration equivalent to half McFarland was prepared using the colonies obtained from cultivation. Then 100 µl of this suspension was added to a petri dish containing Müller-Hilton agar solid medium and was cultured by swapping on the culture medium. Immediately after contaminating the medium with bacterial suspension disking was performed [22]. In this step, 20 µl of each extract concentration, such as 0.15, 0.2, 0.25, and 0.3 mg L^{-1} were added by the sampler and under the biosafety cabinet on paper discs, and the discs were allowed to dry under the biosafety cabinet completely, and then the discs were placed on the culture medium containing the desired bacteria. Eventually, the petri dishes containing the bacterial suspension and disk were placed in the incubator at 36 ºC for 24 h. After 24 h, the transparent halos diameter of growth inhibition around the discs was calculated in mm using a caliper. Discs containing solvent (methanol) and discs prepared with gentamicin antibiotic produced by Padtan Teb Laboratory were applied as positive and negative controls.

Statistical Analysis

Data analysis was done using SAS (9.1) software, and Mean comparisons were performed with Duncan's multiple range test at the 5% significance level. The experimental treatments were implemented factorially, based on a completed blocks randomized design with three replications. The first factor consisted of different nitrogen levels, namely 0, 50, 100, and 150 Kg ha^{-1}, and the second factor was the foliar application of selenium at three levels of 0, 5, and 10 mg L^{-1} of sodium selenate.

RESULTS AND DISCUSSION

Antioxidants

The consequences revealed that the ascorbic acid and antioxidant activity of garlic cloves are increased by selenium and nitrogen. The highest amount of ascorbic acid and antioxidant activity of garlic cloves was achieved when 150 kg ha^{-1} of nitrogen coupled with 10 mg L^{-1} of sodium selenate was used (Table 2).

In opposition to selenium, nitrogen had a positive effect on the content of allicin in garlic. In all four concentrations of nitrogen, with the increasing selenium concentration, the allicin dose decreased. The maximum allicin was seen when 150 kg ha^{-1} of nitrogen along with 0 mg L^{-1} of sodium selenate, which was significantly different from the control treatment. The lowest amount of allicin was demonstrated in the 0 kg ha^{-1} nitrogen treatment with 10 mg L^{-1} of sodium selenate (Table 2).

Table 2 Comparison of the average interaction effect between nitrogen and selenium on antioxidant capacity, ascorbic acid, and allicin of garlic cloves

Nitrogen (Kg ha^{-1})	Selenium (mg L^{-1})	Ascorbic acid (mg 100 g^{-1})	Antioxidant capacity (%)	Allicin (mg g^{-1})
0	0	43.46 i	48.54 i	17.66 gh
	5	47.33 h	52.76 h	17.29 gh
	10	50.43 gh	53.69 gh	16.80 h
50	0	52.93 fg	54.44 fg	19.59 de
	5	55.56 ef	55.24 f	18.75 ef
	10	56.73 ef	56.83 e	18.06 fg
100	0	59.36 de	58.01 de	21.313 ab
	5	61.46 d	58.98 d	20.68 bc
	10	62.83 cd	60.46 c	20.05 b
150	0	65.53 c	61.52 c	21.86 a
	5	71.30 b	63.62 b	22.37 a
	10	79.43 a	66.77 a	21.72 ab

This means that each column of a common letter is significantly different at the 5% level (Duncan's multiple range tests).

Several studies have indicated *Allium* family plants have antimicrobial, anticancer, and antioxidant properties [23 and 24]. An increase in ascorbic acid and antioxidant activity with increasing selenium concentration in onion [25], garlic [26], and turnip (*Brassica rapa* L.) [27] has been reported. Small molecules with sulfur and selenium play a significant role in increasing antioxidant activity [28]. Selenium's antioxidant effect is often related to increasing glutathione peroxidase activity and decreasing lipid peroxidase activity. In many plants, active oxygen is produced in metabolic processes containing photosynthesis and respiration, which may damage chlorophyll, proteins, lipids, and nucleic acids, thereby dormancy the aging, and death of plant cells. However, selenium prevents these problems in plants with its antioxidant role [29]. Selenium increases the antioxidant capacity of plants against active oxygen produced by internal metabolism or external factors [30]. Increased nitrogen concentration was associated with increased ascorbic acid and antioxidant activity of garlic cloves, indicating the role of nitrogen element in increased antioxidant activity, which accords with the results obtained in tomato [31] and rice (*Oryza sativa* L.) [32]. Outcomes exhibited that selenium with nitrogen increased the ascorbic acid and antioxidant activity of garlic cloves, which indicates the two elements positive influence in increasing the antioxidant activity. In addition, when selenium increases the amount of antioxidant combination and enzymes, boosts selenium compound production [33 and 34]. Following the treatment of garlic plants with selenium, that increase in antioxidant activity has a positive effect on ascorbic acid. According to the results of this research, an increase in the amount of ascorbic acid under nitrogen treatment has also been reported in Strawberry (*Fragaria × ananassa* Paros) [35] and lettuce (*Lactuca sativa*) [36]. Nitrogen is a structural component of amino acids, and an expansion in enzyme concentration increases the number of enzymes and antioxidant compounds, which leads to a rise in the amount of antioxidant properties.

Based on the studies, because of its chemical similarity with sulfur, selenium can replace sulfur in the structure of amino acids, which leads to a decrease in the amount of sulfur amino acids. Allicin, one of the most important flavoring compounds in garlic cloves, is influenced by genotype, environmental conditions, fertilization methods, and storage duration. Allicin is a remarkable compound in garlic that is popular in medicine, health, and food [37]. As mentioned earlier, nitrogen can increase the amount of allicin by affecting the absorption of sulfate and selenium, which indirectly affects the antimicrobial activity of garlic extract. However, selenium concentration grows due to the decrease in the sulfur amino acids amount, but the allicin amount in the garlic decreases.

Antibacterial Activity

In the pre-test conducted with concentrations 0.15, 0.2, 0.25, and 0.3 mg L^{-1} of garlic extract, the best outcome in inhibiting the bacteria growth was obtained from the 0.3 mg L^{-1} concentration. Therefore, in the last test, the diameter of the created inhibitory halos was investigated only with a concentration of 0.3 mg L^{-1} of the extract. In order to compare the diameter of the inhibitory halos caused by the application of garlic clove extract, a disk containing 10 µg of gentamicin was used as a positive control and methanol as a negative control (Table 3). Methanol test discs did not show any inhibitory effect on the blossoming of four bacterial strains.

Table 3 Diameter of inhibition halos resulting from the growth of bacteria with the use of gentamicin (10 µg)

Row	The name of the bacterium	Diameter of positive halo (Gentamicin) (mm)
1	*Escherichia coli*	17
2	*Pseudomonas aeruginosa*	15
3	*Bacillus subtilis*	21
4	*Staphylococcus aureus*	20

The existence of only a difference of 2-3 mm in the diameter of the halos can indicate that the target microorganism was sensitive to one drug and resistant to another. If it is resistant, it is concluded that the drug will not affect the bacteria [38]. In recent years, the consumption of garlic has increased due to its taste and benefits to human health. The beneficial properties of garlic are mainly related to the high amount of allicin because the antioxidant, antimicrobial, and anticancer role of compounds has been proven [39].

The diameter of the growth inhibitory halos increased with the increase in the concentration of the extract. Low concentrations (0.1, 0.15, and 0.2 mg L^{-1}) slightly inhibited the growth of bacteria. However, at a high concentration of the extract (0.3 mg L^{-1}), the inhibitory activity of the extract was significant, which was similar to the results obtained by Benkeblia (2004) [40].

Based on the results, with increasing nitrogen and selenium concentrations, the growth inhibition rate of all four bacterial strains increased, and selenium and nitrogen had a positive impact on the inhibition growth rate. The highest and lowest quantity of inhibition of the growth of all four bacteria were observed in the treatments of 150 kg ha^{-1} of nitrogen coupled with 10 mg L^{-1} of sodium selenate and the control, respectively (Table 4).

The antimicrobial activity of garlic extract has been proven against *Escherichia coli*, *Psedomonas paysanans*, *Staphylococcus aureus*, and *Bacillus subtilis* strains [41]. Selenium expanded the antibacterial effect of garlic clove extract, which is consistent with the influences of Bazl (2017) in onion [42]. In garlic, selenium compounds such as seleno-methyl-cysteine, seleno-cysteine, and other selenium compounds play a role in increasing antimicrobial properties [39]. The antibacterial effect of garlic clove increased with the increase of nitrogen level.

According to the research, the formation of selenium compounds plays a main function in the inhibiting impact of bacteria growth. Because selenium, replacing sulfur in sulfur compounds and producing selenium compounds, can increase the inhibiting impact of bacteria growth or intensify the antibacterial influence of sulfate.

Table 4 Comparison of the average interaction effect between nitrogen and selenium on the antibacterial activity of garlic cloves

Nitrogen (Kg ha^{-1})	Selenium (mg L^{-1})	*Escherichia coli*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*
0	0	7.44 j	7.60 k	7.05 j	7.62 j
	5	7.66 ij	8.52 j	7.11 ij	8.33 i
	10	7.87 hi	9.53 i	7.15 i	8.45 hi
50	0	8.03 h	9.82 h	7.30 h	8.52 h
	5	8.11 gh	10.12 g	7.38 gh	8.78 g
	10	8.31f g	10.26 fg	7.44 g	9.07 f
100	0	8.42 ef	10.50 ef	7.59 f	9.36 e
	5	8.61 e	10.72 e	7.82 e	9.48 e
	10	8.84 d	11.27 d	8.07 d	9.75 d
150	0	9.55 c	11.99 c	8.26 c	9.95 c
	5	10.42 b	12.33 b	8.51b	10.21 b
	10	12.00 a	12.73 a	8.95 a	10.90 a

This means that each column of a common letter is significantly different at the 5% level (Duncan's multiple range tests).

CONCLUSION

The methanolic extract of garlic cloves had an inhibitory effect on the bacteria blossoming, and the inhibition rate depended on the methanolic extract concentration and the type of bacteria. The highest amount of antioxidant activity was observed in the highest concentration of nitrogen and selenium. The use of selenium with nitrogen led to an increase in the inhibition consequence on the growth of bacteria. Nitrogen also recreates a role in raising the antibacterial properties by increasing the antioxidant activity. Generally, it can be recommended to employ a concentration of 10 mg L^{-1} of sodium selenate coupled with 150 Kg h^{-1} of nitrogen to produce garlic with maximum antioxidant and antimicrobial properties.

Acknowledgments

The authors are grateful to Razi University and the Agricultural University of Athens.

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