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The use of antioxidant compounds to control root rot disease in peas caused by *Rhizoctonia solani*

Bassam Y. Ibrahim

Department of Plant Protection, College of Agriculture and Forestry, University of Mosul, Ninevah, 41002, Iraq. E-mail: bassamy1966@uomosul.edu.iq

Ban A. Ahmed

*Corresponding author: Department of Pharmacognosy and Medicinal plants, College of Pharmacy, University of Mosul, Ninevah, 41002, Iraq. E-mail: ban-alnuaimy67@uomosul.edu.iq

Firas K. Aljuboori

Department of Plant Protection, College of Agriculture and Forestry, University of Mosul, Ninevah, 41002, Iraq. E-mail: firasaljuboori@uomosul.edu.iq

Abstract

Objective

In order to prevent root rot disease in the crops of peas (*Pisum sativum* L.), this study assessed the impact of the antioxidants citric acid (CA), salicylic acid (SA), and glutathione (GSH) on *Rhizoctonia solani* both in vitro and in vivo.

Materials and Methods

After being extracted from diseased pea roots and purified using the hyphal tip approach, *R. solani* was detected both morphologically and under a microscope. For seven days, root fragments were cultivated on Potato Dextrose Agar (PDA) at 25 ± 2 °C. CA, SA, and GSH were incorporated into PDA in 8.5 cm Petri dishes at concentrations of 0, 50, 100, and 200 mg/L. Fungal colony areas were calculated applying ImageJ software after incubation at 25 °C, and inhibition percentages were calculated. A couple of days before to planting, 3 g biomass kg^{-1} of R. solani was added to sterile soil for in vivo studies. Five replicates of each treatment were planted in pots containing pea seeds. Disease incidence, severity, and post-germination were recorded. Using enzymatic assays, the activities of peroxidase and polyphenol oxidase were measured, and the total phenolic content was computed.

Results

With efficacious concentrations that varied from 50 to 200 mg/L, all antioxidant treatments reduced the growth of *R. solani* in comparison to the control. With a 64% inhibition of fungal

growth in vitro, SA at 200 mg/L showed the most inhibitory impact. SA treatment of seeds significantly decreased the incidence of seed rot and root rot to 8.6% and 45.7%, respectively, and the severity of root rot to 0.21. Antioxidant treatments improved peroxidase, polyphenol oxidase, and total phenolic content in pea plants, with salicylic acid showing the most pronounced enhancement. An undesirable correlation existed between disease incidence and the level of peroxidase, polyphenol oxidase, and total phenolic content

Conclusion

SA revealed greater impacts in boosting enzymatic activities (peroxidase and polyphenol oxidase) and intensifying phenolic compounds in pea plants, successfully lessening *R. solani*-induced root rot. These findings suggested that antioxidant use, solely salicylic acid, provide a positive approach for protecting root rot in peas.

Keywords: Pea plants, *Pisum sativum* L., *Rhizoctonia solani*, Root rot, Salicylic acid

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Introduction

Pea (*Pisum sativum* L.), is one of the important legume crops, has seen marked improves in the formation in recent years (Sari et al., 2021). However, root complaints spoil energetic root role vital for harvest, are turn out to be highly popular (Kumari & Katoch, 2020). Among these, *Rhizoctonia solani*, a extremely critical soil-supported pathogen, impacts multiple crops, holding peas, leading to extensive yield losses. This fungus is accountable for diseases like root rot, stem canker, and damping-off (Naseri, 2013). *R. solani* displays outstanding permanency, remaining viable in soil for long time and resisting sever environmental situations. Plant disease appeared as stunted plant growth and brown to reddish patches on stems and roots, which can sash the stem and

lead to plant death (Quadros et al., 2019). Due to its long host range, protracted soil survival, and genetic diversity, *R. solani* is considered as a major risk to crop yeild (Hane et al., 2014; Ajayi-Oyetunde et al., 2018; Wille et al., 2019). Root rot in peas is repeatedly associated with root rot complex (RRC), which implicates multiple pathogens, containing *R. solani* and several *Fusarium* species (Gossen et al., 2023). Handling this plant infliction associated with deleterious challenges linked to the pathogen's ability to withstand harsh environment in soil for long time. Chemical fungicides can offer effective control (Liu & Khan, 2016); however, their use is prohibited by possibility of environmenta health hazards. Random fungicide use interposes to environmental contamination and carries hazards to human and animal health while negatively impacting favorable soil microorganisms (Tian et al., 2021). Accordingly, there is a persistent requirement to investigates safe and supportable substitutes for disease handling. Antioxidants, like glutathione, SA, and CA, provides positive, eco-friendly options to control *R. solani* due to their safety and effectiveness (Jassim, 2024). This investigation investigates the potential of these antioxidants to manage root rot in peas, providing a foundation for sustainable disease control strategies.

Materials and methods

Chemicals: Chemicals at analytical grade, including CA, SA, and glutathione were purchased from Sigma-Aldrich (USA) were used for this investigation.

Fungal isolation: *Rhizoctonia solani* was extracted from infected pea (*Pisum sativum* L.) roots exhibiting root rot symptoms. Roots were cut into small pieces, surface-sterilized with 1% sodium hypochlorite for 3 minutes, rinsed with sterile distilled water, and placed on 8.5 cm Petri dishes containing Potato Dextrose Agar (PDA). Dishes were incubated at 25 ± 2 °C for 7 days. The fungus was purified applying the hyphal tip technique and identified morphologically based on microscopic and cultural characteristics (Pitt & Hocking, 2009) and macroscopic features (Ogoshi, 1996).

Sample collection: Pea plants (*Pisum sativum* L.) showing symptoms of wilting and yellowing were gathered from vegetable fields at the College of Agriculture and Forestry, University of Mosul, through the 2022–2023 agricultural season. Isolation was carried out from infected roots as described above.

Effect of antioxidants on *Rhizoctonia solani* mycelial growth: CA, SA, and glutathione were incorporated into PDA in 8.5 cm Petri dishes at concentrations of 0, 50, 100, and 200 mg/L.

A 5-mm disk from the edge of an *R. solani* colony was placed at the center of each dish. Plates were incubated at 25 °C for 7 days, and fungal colony areas were calculated applying ImageJ software (version 1.54, NIH, USA). Percentage inhibition was calculated applying the formula:

$$\text{Percentage of inhibition} = \frac{\text{Area of control colony} - \text{Area of treated t colony}}{\text{Area of comparison colony}} x100$$

Pathogenicity test: *Rhizoctonia solani* inoculum was prepared by growing the fungus on millet seeds for 15 days. The fresh (wet) weight of the fungus–seed mixture was applied to inoculate sterile soil at 3 g biomass kg^{-1} dry soil (Lo et al., 1998). Soil was sterilized applying Oxy (Bioglobal, Turkey) at 5 mL/L water, per the manufacturer's instructions, and autoclaved at 121 °C for 20 minutes on two consecutive days. Five pea seeds per 5-kg plastic pot were surface- sterilized with 1% sodium hypochlorite for 3 minutes, rinsed with sterile distilled water, and sown. Germination and seed rot infection percentages were evaluated 2 weeks post-sowing.

Pea seed treatment: Pea seeds were surface-sterilized with 0.1% sodium hypochlorite for 3 minutes, rinsed with sterile distilled water, and soaked for 24 hours in solutions of CA, SA, or glutathione (100 mg/L). Control seeds were soaked in sterile distilled water for 24 hours.

Greenhouse experiment: The effects of CA, SA, and glutathione on *R. solani* root rot were examined in 5-kg sterile plastic pots filled with autoclaved soil (121 °C for 20 minutes on two consecutive days). Two days before planting, soil was inoculated with *R. solani* grown on PDA at 3 g biomass kg^{-1} soil. Five replicates per treatment were applied, with five pea seeds sown per pot. Post-germination, the subsequent parameters were documented: percentage fungal inhibition, root length, root fresh and dry weight, shoot length, shoot fresh and dry weight, and root rot infection rate. The percentage of infection was calculated as:

Percentage of infection = (number of infected plants) / (total number of plants) x 100

Root rot severity was evaluated applying the subsequent scale (Dorrance et al., 2003): 0 = Healthy roots; 1 = 1–33% spotted roots; 2 = 34–50% spotted roots; 3 = 51–80% spotted roots.

Growth parameters: Root and shoot lengths (cm), fresh and dry weights of roots and shoots (g), and relative leaf chlorophyll content (SPAD units) were calculated applying a chlorophyll meter (Minolta, Japan) (Ling et al., 2011).

Biochemical assays: The enzyme activity of Peroxidase (POD) and Polyphenol Oxidase (PPO) was calculated applying the filtrate of 1 g of pea roots and 10 milliliters of 0.1M sodium phosphate buffer (pH 7) after they were homogenized and centrifuged at 28 G for 20 minutes (Kukavica *et al*., 2012). Activity of peroxidase (POD) was calculated in accordance with (Howell, 2003). The Folin–Ciocalteu reagent was applied to detect total phenols in accordance with Singleton et al. (1999). Peroxidase (POD) and polyphenol oxidase (PPO) activities were calculated applying crude enzyme extracts from 0.5 g of pea leaves ground in 10 mL of pre-

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chilled 0.1 M potassium phosphate buffer (pH 7.0). The homogenate was filtered through Whatman No. 1 filter paper and centrifuged at 4000 rpm for 10 minutes applying a 14-cm rotor. The supernatant was stored at 4 °C until analysis (Pitotti et al., 1994). Total phenolic content was identified applying the Folin-Ciocalteu reagent (Singleton et al., 1999).

Peroxidase activity: POD activity was calculated subsequent Müftügil (1985). A 2-mL reaction mixture containing 1 mL of 1.0% hydrogen peroxide and 1 mL of 0.54% guaiacol was mixed with 0.1 mL of enzyme extract in a spectrophotometer cuvette. Absorbance at 420 nm was documented every 60 seconds for 3 minutes. Enzyme activity (U/mL) was calculated as:

Enzyme activity (U/min) = ΔA/ΔTx0.01

where ΔA (Delta A) is the alter in absorbance at 420 nm, and Δt (Delta t) is time in minutes. One unit of enzyme activity is defined as the amount creating a 0.01 absorbance improve per minute at 420 nm.

Polyphenol oxidase activity: PPO activity was calculated subsequent Hamza (2012). A 2-mL reaction mixture containing 1 mL of 0.2 M potassium phosphate buffer (pH 7.0) and 1 mL of 0.02 M catechol was mixed with 1 mL of enzyme extract in a spectrophotometer cuvette. Absorbance at 420 nm was documented every 60 seconds for 3 minutes. Enzyme activity (U/mL) was calculated applying the same formula as for POD.

Statistical analysis: A randomized complete block design (RCBD) with five replicates was applied. Data were analyzed applying GraphPad Prism (version 10, GraphPad Software, USA), and means were compared applying the least meaningful difference (LSD) test at a 0.05 probability level.

Results and Discussion

Effect of CA, SA, and glutathione on inhibiting the growth of *R. solani:* As shown in Figure 1, salicylic acid (SA) meaningfully prevented the growth of *R. solani* in PDA medium, with inhibition percentages of 48.2%, 56.5%, and 64.8% at concentrations of 50, 100, and 200 mg/L, respectively. In contrast, citric acid (CA) and glutathione exhibited minimal inhibitory effects. These finding are in congruent with Dieryckx et al. (2015), who have reported that using PDA medium with SA at dilution limits 1 to 25 mM blocked fungal mycelial multiplication. Furthermore, SA is recognized to jeopardize fungal cell membranes, leading to cell death and consequent growth inhibition (Kong et al., 2021).

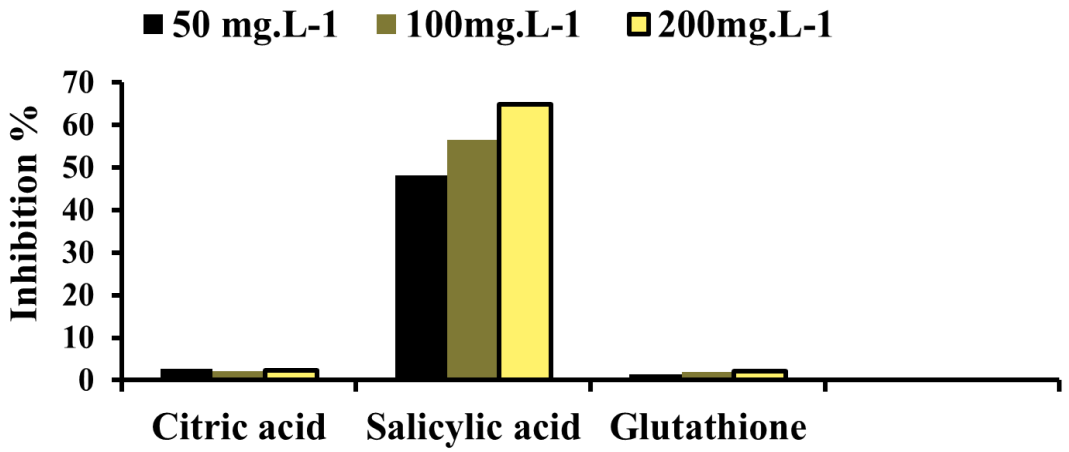

Figure 1. Inhibitory effect of SA, CA, and glutathione treatments on the mycelial growth of *R. solani* in vitro

Effect on seed and root rot incidence: Soaking pea seeds with SA markedly inhibited the occurrence of seed and root rot parallel to that of CA and glutathione. Moreover, the SA exposure ensued in 8.6% seed rot and 45.7% root rot, with root rot severity diminished to 0.12 (Table 1). These findings are experimentally in congruent with Mohamed and Amer (2014), who highlighted that SA exposure markedly blunted both damping-off and root rot in squash and cantaloupe, in addition to flourishing the plant survival incidences.

Table 1. Effect of CA, SA, and glutathione on the incidence and severity of seed and root rot

Treatments	seed rot (%)	Root rot (%)	Root rot severity
CA	12.4	61.2	0.23
SA	8.6	45.7	0.12
Glutathione	10.2	51.7	0.29
Control	35.2	85.4	0.81
L.S.D.	10.2	4.7	0.19

Effect on growth parameters in plants infected with *R. solani:* CA, SA, and glutathione treatments have induced marked inhibition in growth parameters of shoot and root in plants exposed to *R. solani*, the shoot length was most rigorously affected by SA, with a reduction to 9.64 units, followed by CA (10.42 units) and glutathione (10.21 units) versus 53.2 units in the control group (Table 2). The least meaningful difference (LSD) for shoot length was 4.42 units. Similarly, the root length followed the same pattern, the control plants shown roots of 81.4 units,

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while SA, CA, and glutathione treatments resulted in 12.28, 15.95, and 15.63 units, respectively (LSD= 2.12 units). The fresh weight for shoots and roots also demonstrated notable reductions. The fresh weight of the control shoot and root were 65.5 and 71.8 units, respectively. However, the SA-exposed groups demonstrated reduced measurement (12.1 and 11.56 units, respectively). The LSDs for shoot and root fresh weights were 2.19 and 1.46 units, respectively. Dry weight patterns were in line with fresh weight data. SA demonstrated the most prominent effects, reducing shoot and root dry weights to 6.34 and 11.66 units, respectively. The control group reported 41.7 and 78.3 units, with LSDs of 1.72 and 2.13 units.

Table 2. Effect of CA, SA, and glutathione on shoot and root length, fresh weight, and dry weight

	Length		Wet weight		Dry weight	
Treatments	Shoot	Root	Shoot	Root	Shoot	Root
CA	10.42	15.95	16.95	13.28	8.17	15.34
SA	9.64	12.28	12.1	11.56	6.34	11.66
Glutathione	10.21	15.63	13.81	13.02	8.02	15.03
Control	53.2	81.4	65.5	71.8	41.7	78.3
LSD	4.42	2.12	2.19	1.46	1.72	2.13

LSD=Least Significant Difference, CA= Citric acid, SA=Salicylic acid

These findings harmonized with Jassim (2024), who reported that SA usage enhance root and shoot growth and encourage chlorophyll content.

Impact on total phenols and enzyme activity (peroxidase and polyphenol oxidase): Marked rise in enzymatic action and total phenolic content were detected ensuing handlings with CA, SA, and glutathione versus the control group. The SA exposure associated with the increasing polyphenol oxidase (PPO) activity (101.14 units/min/g fresh weight), followed by CA (94.75) and glutathione (89.78), with the control at 75.42. The LSD was 10.2 units/min/g. Peroxidase activity demonstrated same trends. SA and CA application implicated in 62.74 and 63.25 units/min/g, respectively, while glutathione produced a moderate convalesce to 45.73 (control = 41.91; LSD = 4.7).

Total phenol content was also markedly increased in SA-exposed plants accumulated 6.74 mg/g, followed by CA (6.13 mg/g) and glutathione (5.49 mg/g), while

the control was at 2.14 mg/g (LSD = 0.19 mg/g).

Table 3. Effect of CA, SA, and glutathione on polyphenol oxidase, peroxidase activity, and total phenolic content

	Polyphenol oxidase (uint.min g fw^{-1})	Peroxidase (uint.min g fw^{-1})	Total phenols (mg. g. fw^{-1})
CA	94.75	63.25	6.13
SA	101.14	62.74	6.74
Glutathione	89.78	45.73	5.49
Control	75.42	41.91	2.14
LSD	10.2	4.7	0.19

LSD=Least Significant Difference, CA= Citric acid, SA=Salicylic acid

Considering the three approaches of usage, SA was the most powerful stimulant of PPO action, demonstrating a 34% recovery versus the control. Perhaps this potentially could be explained in the context of the hormonal impact of SA that elicits systemic acquired resistance (SAR) approaches, augmenting the expression of defense-related genes. Moreover, CA revealed marked value, upregulating PPO effectiveness by 25.6%, perhaps by adjusting plant metabolic activities and potentiating defense enzymes. One of these effectively involved defense antioxidant enzymes is glutathione, demonstrated the lowest improve (19%) in PPO action among the used approaches of treatments. Moreover, there are reportedly increased peroxidase activity by SA and CA reaching up to ~50% improvement compared to control, reflecting that they may impart parallel mechanisms in inducing oxidative stress responses. One of these enzymes was glutathione stimulated a 9% enhanced, suggesting alternative mechanism of action.

Additionally, SA induced the highest buildup of total phenolics (215% improve), tracked by CA (186%) and glutathione (157%) versus control, reflecting that this demonstrated that SA effective role in encouraging antioxidant defenses enzymes and phenolic compound biosynthesis. These findings are reinforced by former researches (Khalil et al., 2018; Ali et al., 2023; Yin et al., 2024; Ali et al., 2024), which demonstrated that the role of SA in enhancing antioxidant enzyme effects and presenting the post-harvest shelf life. At the molecular level, Yin et al. (2024) reflected that SA therapy through barley germination markedly enhanced total phenolic acids. Moreover, SA augmented peroxidase, polyphenol oxidase, and catalase activities within 12–24 hours of *Magnaporthe oryzae* inoculation. Thepbandit et al. (2024)

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reported that SA-treated plants revealed lowered disease symptoms and reduced blast severity in rice under greenhouse standards.

Conclusions: In this study, SA displayed valuable biocontrol action under greenhouse standard culture protocol, suggesting its role as a potential agent for managing *Rhizoctonia* root rot in peas. Moreover, SA worked as a plant growth enhanced by boosting antioxidant enzyme effects, hence reducing reactive oxygen species levels and membrane lipid peroxidation. These physiological effects contribute to improved resistance against clubroot disease in peas.

Author contributions

Concept of the study: BYI and BAA; Result collection: BYI and BAA; Data analysis: FKDA; Methodology: BYI and BAA; Visualization: FKDA; Writing-original draft: BYI and BAA; Writing-review & editing: FKDA. All authors participated equally to the concept and the writing of the original and subsequent drafts.

Data availability statement

Available with the corresponding authors on request.

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Ethical considerations

Not applicable

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Conflict of interest

No conflict of interest existed in this research.

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مجله بیوتکنولوژی کشاورزی
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انجمن بیوتکنولوژی ایران

استفاده از ترکیبات آنتی‌اکسیدان برای کنترل بیماری پوسیدگی ریشه در نخود فرنگی ناشی از *Rhizoctonia solani*

بسام ی. ابراهیم

گروه حفاظت گیاه، دانشکده کشاورزی و جنگلداری، دانشگاه موصل، نینوا، ۴۱۰۰۲، عراق. ایمیل: bassamy1966@uomosul.edu.iq

بان ع. احمد

نویسنده مسئول: گروه فارماکوگنوزی و گیاهان دارویی، دانشکده داروسازی، دانشگاه موصل، نینوا، ۴۱۰۰۲، عراق. ایمیل: ban-alnuaimy67@uomosul.edu.iq

فراس ک. الجبوری

گروه حفاظت گیاه، دانشکده کشاورزی و جنگلداری، دانشگاه موصل، نینوا، ۴۱۰۰۲، عراق. ایمیل: firasaljuboori@uomosul.edu.iq

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چکیده

هدف: این مطالعه اثر آنتی‌اکسیدان‌های اسید سیتریک (CA)، اسید سالیسیلیک (SA) و گلوتاتیون (GSH) را به‌منظور کنترل بیماری پوسیدگی ریشه در گیاه نخود فرنگی (*Pisum sativum* L.)، به‌صورت برون گیاهی (in vitro) و درون‌گیاهی (in vivo) بر روی قارچ *Rhizoctonia solani* مورد ارزیابی قرار داد.

مواد و روش‌ها: قارچ *Rhizoctonia solani* از ریشه‌های آلوده نخود جدا شد و با استفاده از روش نوک هیف خالص‌سازی و به صورت ریخت‌شناسی و میکروسکوپی شناسایی گردید. قطعات ریشه روی محیط کشت PDA کشت داده شده و به مدت ۷ روز در دمای ۲±۲۵ درجه سانتی‌گراد انکوبه شدند. آنتی‌اکسیدان‌های CA، SA و GSH به محیط PDA در پلیت‌های ۸/۵ سانتی‌متری در غلظت‌های ۰، ۵۰، ۱۰۰ و ۲۰۰ میلی‌گرم در لیتر اضافه شدند. پس از انکوباسیون در دمای ۲۵ درجه، مساحت کلونی‌های قارچی با نرم‌افزار ImageJ اندازه‌گیری و درصد مهار رشد محاسبه گردید. در آزمایش‌های درون‌گیاهی، خاک استریل با ۳ گرم زیست‌توده قارچ در هر کیلوگرم خاک، دو روز پیش از کاشت بذرها تلقیح شد. بذرهای نخود در گلدان‌هایی با پنج تکرار برای هر تیمار کاشته

شدند. پس از جوانه‌زنی، درصد وقوع و شدت بیماری ثبت شد. فعالیت آنزیم‌های پراکسیداز و پلی‌فنل اکسیداز با آزمون‌های آنزیمی اندازه‌گیری شد و میزان ترکیبات فنلی کل نیز تعیین گردید.

نتایج: تمام تیمارهای آنتی‌اکسیدانی رشد قارچ *R. solani* را نسبت به شاهد مهار کردند. غلظت‌های مؤثر بین ۵۰ تا ۲۰۰ میلی‌گرم در لیتر بودند. اسید سالیسیلیک با غلظت ۲۰۰ میلی‌گرم در لیتر بیشترین اثر مهاری را با ۶۴٪ مهار رشد قارچ در شرایط درون‌گیاهی نشان داد. تیمار بذر با اسید سالیسیلیک به‌طور معناداری پوسیدگی بذر و ریشه را به ۸/۶ و ۴۵/۷ درصد و شدت بیماری را به ۰/۲۱ کاهش داد. تیمارهای آنتی‌اکسیدانی باعث افزایش فعالیت آنزیم‌های پراکسیداز، پلی‌فنل اکسیداز و ترکیبات فنلی کل در گیاهان نخود شدند و اسید سالیسیلیک بیشترین افزایش را نشان داد. رابطه معکوسی بین بروز بیماری و فعالیت آنزیم‌های مذکور و میزان ترکیبات فنلی مشاهده شد.

نتیجه‌گیری: اسید سالیسیلیک عملکرد بالاتری در افزایش فعالیت آنزیم‌های پراکسیداز و پلی‌فنل اکسیداز و افزایش ترکیبات فنلی در گیاه نخود نشان داد و به طور مؤثری باعث کاهش پوسیدگی ریشه ناشی از *R. solani* گردید. این نتایج نشان می‌دهند که استفاده از آنتی‌اکسیدان‌ها، به‌ویژه اسید سالیسیلیک، روشی امیدوارکننده برای مدیریت بیماری پوسیدگی ریشه در نخود فرنگی است.

کلمات کلیدی: اسید سالیسیلیک، پوسیدگی ریشه، نخود فرنگی، *Pisum sativum* L.، *Rhizoctonia solani*

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