

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

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Abstract

Objective

This investigation evaluated the effects of the antioxidants citric acid (CA), salicylic acid (SA), and glutathione (GSH) on *Rhizoctonia solani* in vitro and in vivo to control root rot disease in pea plants (*Pisum sativum* L.).

Materials and Methods

Rhizoctonia solani was extracted from infected pea roots, purified applying the hyphal tip technique, and identified morphologically and microscopically. Root fragments were cultured on Potato Dextrose Agar (PDA) and incubated at 25 ± 2 °C for 7 days. 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. For in vivo experiments, sterile soil was inoculated with *R. solani* at 3 g biomass kg⁻¹ soil, two days before planting. Pea seeds were sown in pots with five replicates per treatment. Post-germination, disease incidence and harshness were documented. Peroxidase and polyphenol oxidase activities were quantified applying enzymatic assays, and total phenolic content was calculated.

Results

All antioxidant treatments inhibited *R. solani* growth compared to the control, with effective concentrations ranging from 50 to 200 mg/L. Salicylic acid at 200 mg/L exhibited the highest

inhibitory effect, achieving 64% inhibition of fungal growth in vitro. Seed treatment with salicylic acid meaningfully reduced seed rot and root rot incidence to 8.6% and 45.7%, respectively, and root rot severity to 0.21. Antioxidant treatments improved peroxidase, polyphenol oxidase, and total phenolic content in pea plants, with salicylic acid showing the most pronounced enhancement. A negative correlation was observed between disease incidence and the activities of peroxidase, polyphenol oxidase, and total phenolic content.

Conclusion

Salicylic acid demonstrated superior effectiveness in enhancing enzymatic activities (peroxidase and polyphenol oxidase) and increasing phenolic compounds in pea plants, effectively reducing *R. solani*-induced root rot. These results propose that antioxidant treatments, exclusively salicylic acid, offer a hopeful approach for managing 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.), the second most important legume crop globally, has seen meaningful improves in production in recent years (Sari et al., 2021). However, root diseases, which impair vital root functions essential for yield, are becoming increasingly prevalent (Kumari & Katoch, 2020). Among these, *Rhizoctonia solani*, a highly destructive soil-borne pathogen, affects multiple crops, containing peas, creating substantial yield losses. This fungus is responsible for diseases like root rot, stem canker, and damping-off (Naseri, 2013). *R. solani* exhibits remarkable persistence, staying viable in soil for extended periods and withstanding harsh environmental conditions. Disease symptoms include stunted plant growth and brown to reddish lesions on stems and roots, which can girdle the stem and lead to plant death (Quadros et al., 2019). Due to its broad host range, prolonged soil survival, and genetic diversity, *R. solani* is

attended a major threat to crop productivity (Hane et al., 2014; Ajayi-Oyetunde et al., 2018; Wille et al., 2019). Root rot in peas is often related to the root rot complex (RRC), which involves multiple pathogens, containing *R. solani* and numerous *Fusarium* species (Gossen et al., 2023). Managing this disease shows meaningful challenges due to the pathogen's capability to survive saprophytically in soil for extended periods. Chemical fungicides can supply effective control (Liu & Khan, 2016); however, their applying is restricted by potential health and environmental risks. Indiscriminate fungicide application contributes to environmental contamination and poses threats to human and animal health while adversely affecting beneficial soil microorganisms (Tian et al., 2021). Consequently, there is a pressing need to identify safe and sustainable alternatives for disease management. Antioxidants, like glutathione, salicylic acid, and citric acid, offer hopeful, eco-friendly approaches 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: Citric acid (CA), salicylic acid (SA), and glutathione (all from Sigma-Aldrich, St. Louis, MO, USA) were applied in this investigation. All chemicals were of analytical grade.

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:

$$\text{Percentage of inhibition} = \frac{\text{Area of control colony} - \text{Area of treated t colony}}{\text{Area of comparison colony}} \times 100$$

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:

$$\text{Percentage of infection} = (\text{number of infected plants}) / (\text{total number of plants}) \times 100$$

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-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:

$$\text{Enzyme activity (U/min)} = \Delta A / \Delta T \times 0.01$$

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 results are in line with Dieryckx et al. (2015), who announced that supplementing PDA medium with SA concentrations ranging from 1 to 25 mM prevented fungal mycelial growth. Additionally, SA is known to disrupt fungal cell membranes, leading to cell death and subsequent growth inhibition (Kong et al., 2021).

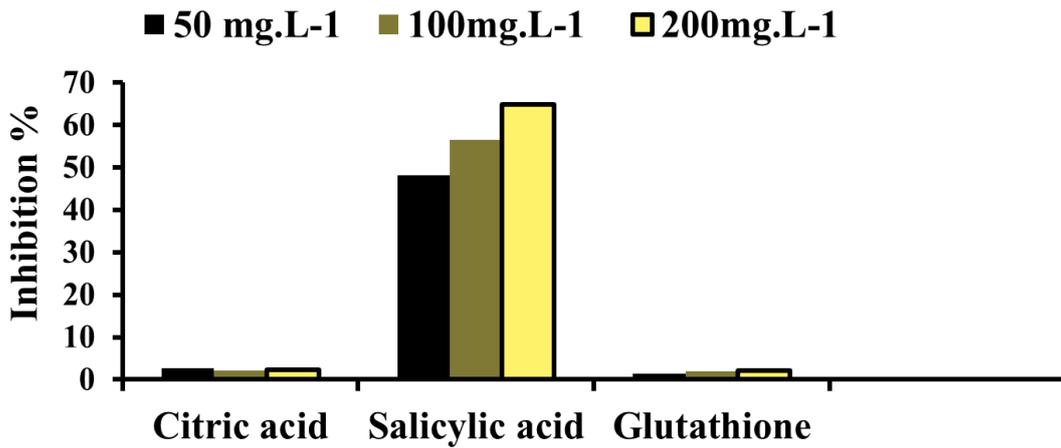


Figure 1. Inhibitory effect of SA, CA, and glutathione treatments on the mycelial growth of *R. solani* in vitro

Effect on seed and root rot incidence: Table 1 shows that soaking pea seeds with SA meaningfully reduced the incidence of seed and root rot compared to CA and glutathione. The SA treatment resulted in 8.6% seed rot and 45.7% root rot, with root rot severity reduced to 0.12. These results are in line with Mohamed and Amer (2014), who announced that salicylic acid treatments meaningfully reduced both damping-off and root rot in squash and cantaloupe, in addition to improving plant survival rates.

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*: Data in Table 2 demonstrate meaningful reductions in shoot and root growth parameters due to CA, SA, and glutathione treatments in plants exposed to *R. solani*. Shoot length was most severely impacted by SA, with a reduction to 9.64 units, followed by CA (10.42 units) and glutathione (10.21 units) compared to the control (53.2 units). The least meaningful difference (LSD) for shoot length was 4.42 units. Root length showed a similar trend. Control plants developed roots of 81.4 units, while

SA, CA, and glutathione treatments resulted in 12.28, 15.95, and 15.63 units, respectively (LSD = 2.12 units). Fresh weight measurements for shoots and roots also showed meaningful reductions. Control shoot and root fresh weights were 65.5 and 71.8 units, while SA-treated plants showed the lowest values (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 showed the most pronounced effects, reducing shoot and root dry weights to 6.34 and 11.66 units, respectively. The control group documented 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

Treatments	Length		Wet weight		Dry weight	
	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 results are supported by Jassem (2024), who found that salicylic acid application improved root and shoot development and improved chlorophyll content.

Impact on total phenols and enzyme activity (peroxidase and polyphenol oxidase): Meaningful differences in enzymatic activity and phenolic content were observed subsequent treatments with CA, SA, and glutathione compared to the control. SA treatment led to the highest 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 followed a similar pattern. SA and CA treatments resulted in 62.74 and 63.25 units/min/g, respectively, while glutathione generated a moderate improve to 45.73 (control = 41.91; LSD = 4.7).

Total phenol content was also meaningfully elevated. SA-treated 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 ($\mu\text{int}\cdot\text{min g fw}^{-1}$)	Peroxidase ($\mu\text{int}\cdot\text{min g fw}^{-1}$)	Total phenols ($\text{mg}\cdot\text{g}\cdot\text{fw}^{-1}$)
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

Among the three treatments, SA was the most potent inducer of PPO activity, showing a 34% improve compared to the control. This may be due to its role as a plant hormone that triggers systemic acquired resistance (SAR) pathways, enhancing the expression of defense-related genes. CA also showed meaningful effectiveness, increasing PPO activity by 25.6%, likely by modulating plant metabolic processes and activating defense enzymes. Glutathione, while effective, showed the lowest improve (19%) in PPO activity among the treatments. Peroxidase activity was similarly improved by SA and CA (~50% improve vs control), denoting they may share similar mechanisms in stimulating oxidative stress responses. Glutathione induced a 9% improve, proposing a different mode of action. SA also resulted in the highest accumulation of total phenolics (215% improve), followed by CA (186%) and glutathione (157%) compared to control. This highlights SA’s meaningful role in enhancing antioxidant defenses and phenolic biosynthesis. These results are supported by previous investigations (Khalil et al., 2018; Ali et al., 2023; Yin et al., 2024; Ali et al., 2024), which report the role of SA in improving antioxidant enzyme activity and extending post-harvest shelf life. At the molecular level, Yin et al. (2024) demonstrated that salicylic acid treatment through barley germination meaningfully improved total phenolic acids. Additionally, SA enhanced peroxidase, polyphenol oxidase, and catalase activities within 12–24 hours of *Magnaporthe oryzae* inoculation. Thepbandit et al. (2024) further announced that SA-treated plants exhibited reduced disease symptoms and lower blast severity in rice under greenhouse conditions.

Conclusions: In the current investigation, salicylic acid (SA) demonstrated effective biocontrol activity under greenhouse conditions, proposing its potential as a hopeful agent for managing *Rhizoctonia* root rot in peas. Additionally, SA acted as a plant growth promoter by enhancing antioxidant enzyme activities, thereby reducing reactive oxygen species (ROS) levels

and membrane lipid peroxidation. These physiological effects contribute to improved resistance against clubroot disease in peas.

Author contributions

Concept of the investigation: BYI and BAA; Data collection: BYI and BAA; Result analysis: FKDA; Investigation: BYI and BAA; Methodology: BYI and BAA; Project supervision: FKDA; Software: FKDA; Resources: BYI and BAA; Validation: FKDA; 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

Data is available on request from the corresponding authors.

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Ethical considerations

Not applicable

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Conflict of interest

The authors declare no conflict of interest.

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

از *Rhizoctonia solani*

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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 برون‌گیاهی (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 25 ± 2 درجه سانتی‌گراد انکوبه شدند. آنتی‌اکسیدان‌های SA، CA، GSH به محیط کشت 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. بذرها 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 به‌طور 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، *Rhizoctonia solani*, *Pisum sativum* L.

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