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Apigenin and apigenin-7-O- β -d-glucoside enhance pepper defense against *Phytophthora capsici* by inhibiting pathogen growth and reproduction

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Abstract

Apigenin is a natural plant flavonoid known for its remarkable biological activity. While numerous studies have documented the accumulation of glycosylated and non-glycosylated apigenin in fruit and vegetable crops, apigenin's direct and indirect effects on plant resistance remain poorly understood. This study employed non-targeted metabolomics strategies to monitor metabolome-reprogramming events in susceptible, moderately resistant, and highly resistant pepper (*Capsicum annuum*) cultivars challenged with *Phytophthora capsici*. Results revealed a positive correlation between the accumulation of apigenin and apigenin-7-O- β -d-glucoside in pepper seedlings inoculated with *P. capsici* and the resistance characteristics of the individual pepper cultivars. Further, we demonstrated that both apigenin and apigenin-7-O- β -d-glucoside significantly inhibited the vegetative growth of economically important phytopathogenic oomycetes and fungi, including *P. capsici*, *P. sojae*, *Pyricularia oryzae*, *Fusarium graminearum*, and *Colletotrichum siamense*. These findings highlight the potential applications of glycosylated and non-glycosylated apigenin in developing biopesticides to support the environment-friendly prevention and control of critical crop fungal and oomycete diseases.

Keywords Plant immunity, Apigenin-7-O- β -d-glucoside, Apoptosis, Pathogen-host interaction, Green disease management

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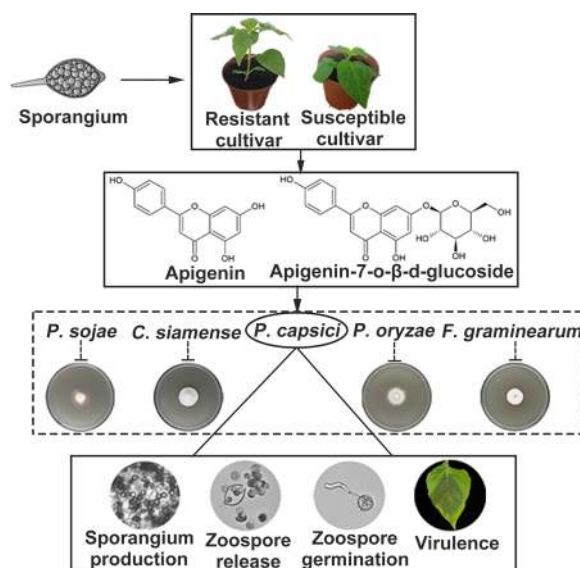
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Graphical abstract



Background

Phytophthora capsici is a destructive soil-borne phytopathogenic oomycete capable of infecting all foliar tissues, including leaves, fruits, and stems/vines, causing diverse diseases such as damping-off, crown rot, foot rot, dieback, phytophthora blight, root rot, fruit rot, and wilting in 78 plant species from 18 families. Hosts of *P. capsici* include economically significant fruits and vegetables, such as pepper, eggplant, watermelon, cucumber, squash, snap bean, soybean, lima bean, cantaloupe, and pumpkin (Hausbeck and Lamour 2004; Quesada-Ocampo et al. 2023). *P. capsici* infections account for crop losses valued at \$1 billion annually (Lamour et al. 2012). Environmental conditions, including warm temperatures, high humidity, rainy weather, and improper irrigation, accelerate the dissemination, initiation, and development of *Phytophthora* blight caused by *P. capsici* (Mondal et al. 2020).

Current control strategies for *P. capsici* rely heavily on synthetic chemicals and cultural farming practices, including crop rotation, proper spacing, mixed cropping, farm hygiene, enhanced soil drainage, proper tillage, and irrigation (Retes-Manjarrez et al. 2020; Wang et al. 2022). However, these cultural practices are often less effective than synthetic oomycides (fungicides), such as benzoylamide, metalaxyl, and mandipropamid. Chemical control measures, however, tend to increase production costs and negatively impact the environment and human health (Kousik et al. 2011; Naqvi

et al. 2024). Further, prolonged and intensive use of chemicals promotes the development of drug resistance among pathogenic microbes, including field isolates of *P. capsici* (Tyers and Wright 2019; Lee et al. 2023; Ishii 2024). Attempts to breed resistance in plants against this broad-host pathogen have not yielded durable success due to significant variation in virulence and tissue preference among *P. capsici* field isolates. Studies have also revealed that *P. capsici* undergoes frequent genome reshuffling to overcome inbred resistance in host plants (Kamoun et al. 2015; Ro et al. 2022).

In addition to external interventions, plants possess and readily deploy genetic and chemical defenses to thwart the initiation of the parasitic process by invading pathogens. The contributions of genetic parameters in host plants, including genes coding for pathogenesis-related (PR) or resistance-associated proteins, such as CaWRKY01-10 and CaWRKY08-4, ascorbate peroxidase, β-1,3-glucanases, aquaporins, late blight resistance protein (CA11g02470), and PR protein (CA04g13070), to either complete or partial resistance of selected fruit and vegetable crops (particularly in pepper cultivars against *P. capsici*) have attracted considerable research attention (Cheng et al. 2024; Shi et al. 2024). However, the contributions of plant-specific metabolites (phytochemicals) derived from *P. capsici*-induced metabolome reprogramming to the resistance of pepper cultivars against blight pathogens are not fully understood.

Classes of plant metabolites reported to accumulate in host tissues or infection sites include terpenes

(monoterpenes, sesquiterpenes, diterpenes, and polyterpenes), phenolics (flavonoids, isoflavonoids, tannins, coumarin, etc.), and nitrogen-containing compounds (alkaloids), inter alia (Jan et al. 2021). Flavonoids constitute a predominant proportion of plant secondary metabolites (Górniak et al. 2019). Flavonoids, including apigenin, anthocyanins, cyanidin, caffeic acid, chlorogenic acid, and daidzin, represent essential antioxidant and antimicrobial compounds with significant pharmacological value (Chagas et al. 2022). Recent research has revealed that differences in the accumulation of caffeic acid and chlorogenic acid in ripe and unripe pepper fruits during anthrax infection determine pepper's resistance to anthrax (Baba et al. 2019).

Although studies have identified apigenin and apigenin-7-O- β -d-glucoside, natural plant flavonoids found in celery (*Apium graveolens*), parsley (*Petroselinum crispum*), and other fruits and vegetables (Williams and Grayer 2004; Petrusa et al. 2013; Tang et al. 2017), with significant anticancer, antioxidant, anti-inflammatory, antibacterial, and antiviral properties (Clavin et al. 2007; Benavente-Garcia and Castillo 2008; Rossato et al. 2011), the inherent contributions of glycosylated and non-glycosylated apigenin to plant defense against phytopathogenic microbes, including *P. capsici*, are just beginning to attract research attention.

Results from comparative metabolome profiling of *P. capsici*-susceptible, moderately resistant, and resistant pepper cultivars challenged with *P. capsici* revealed an increasing accumulation of apigenin and apigenin-7-O- β -d-glucoside that correlated with the resistance capabilities of the individual pepper cultivars. Further, we demonstrated that both apigenin and apigenin-7-O- β -d-glucoside significantly inhibited the vegetative growth of economically important phytopathogenic oomycete and fungal species, including *P. capsici*, *P. sojae*, *Pyricularia oryzae*, *Fusarium graminearum*, and *Colletotrichum siamense*. This study provides crucial insights into the antimicrobial efficacy of apigenin and apigenin-7-O- β -d-glucoside, underscoring the potential of glycosylated and non-glycosylated apigenin in developing biopesticides to support the environment-friendly prevention and control of essential plant pathogenic oomycete and fungal pathogens.

Results

Accumulation of glycosylated and non-glycosylated apigenins in resistant pepper seedlings in response to *P. capsici* infection

To explore the possible connection between the accumulation of apigenin and apigenin-7-O- β -d-glucoside and resistance variations in different pepper cultivars (HNUCB0081, HNUCB0226, and XIAO ZHOU PI)

against *P. capsici*, we analyzed non-targeted metabolomics data from samples collected at different stages of *P. capsici*-pepper interactions. The results revealed a significant increase in apigenin and apigenin-7-O- β -d-glucoside levels in the completely resistant (XIAO ZHOU PI) and moderately resistant (HNUCB0226) cultivars, compared to the highly susceptible HNUCB0081 cultivar in response to *P. capsici* infection (Fig. 1a, b, and Additional file 1: Figure S1).

We quantitatively analyzed apigenin and apigenin-7-O- β -d-glucoside levels in inoculated leaf tissues of HNUCB0081, HNUCB0226, and XIAO ZHOU PI seedlings under *P. capsici*-induced biotic stress using targeted metabolomics. Our analyses revealed a significant increase in intracellular apigenin levels, particularly in the HNUCB0081 and HNUCB0226 cultivars (Fig. 1c, e). Unexpectedly, the intracellular levels of apigenin-7-O- β -d-glucoside in XIAO ZHOU PI before inoculation were significantly higher than in both the inoculated and control groups of HNUCB0081 and HNUCB0226. During *P. capsici* infection, apigenin-7-O- β -d-glucoside levels in XIAO ZHOU PI were significantly higher than in HNUCB0081 and HNUCB0226 under the same conditions (Fig. 1d, f).

Apigenin and apigenin-7-O- β -d-glucoside inhibit the vegetative growth of phytopathogens, including *P. capsici*

We investigated the inhibitory effects of apigenin and apigenin-7-O- β -d-glucoside on *P. capsici* by assessing the vegetative growth of LT1534 strains on TA agar plates supplemented with different concentrations of apigenin or apigenin-7-O- β -d-glucoside. Compared to the control groups, we observed a significant reduction in the vegetative growth of *P. capsici* on media containing either compound, with stable IC_{50} values of 126.9 μ M and 110.6 μ M, respectively (Fig. 2a, b, d, e, g, h). These results confirm that both apigenin and apigenin-7-O- β -d-glucoside significantly inhibit the vegetative growth of *P. capsici*.

Additionally, we examined the inhibitory effects of a combination of apigenin and apigenin-7-O- β -d-glucoside at a 1:1 molar ratio on TA plates. At concentrations of 5 μ M or higher, vegetative growth was inhibited; at 150 μ M, the inhibition was almost complete (Fig. 2c, f). The IC_{50} values calculated for apigenin, apigenin-7-O- β -d-glucoside, and their combination demonstrated that the combined treatment exerted a higher inhibitory effect on *P. capsici* growth, with an IC_{50} of 35.63 μ M compared to 126.9 μ M and 110.6 μ M, for apigenin and apigenin-7-O- β -d-glucoside alone. These findings suggest that the moderate accumulation of these compounds plays a crucial role in enhancing the resistance of pepper cultivars against *P. capsici*.

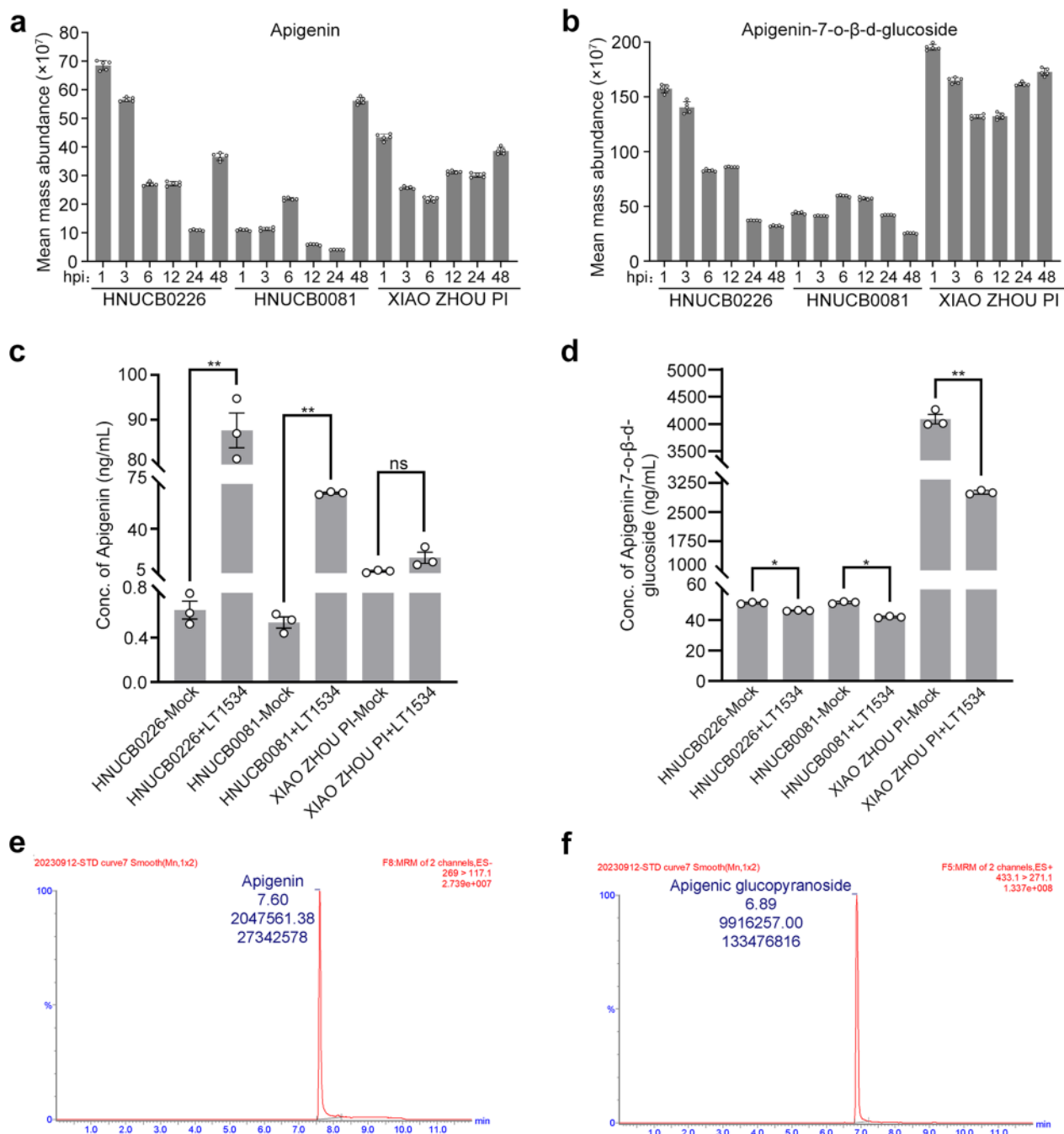


Fig. 1 Relative quantification of apigenin and apigenin-7-O-β-d-glucoside in different pepper cultivars challenged with *P. capsici*. **a, b** Comparative abundance of apigenin and apigenin-7-O-β-d-glucoside in susceptible and resistant pepper cultivars at different stages of *P. capsici* infection. **c, d** Targeted metabolomics quantification of apigenin and apigenin-7-O-β-d-glucoside concentrations in the susceptible and resistant pepper cultivars during *P. capsici* infection. **d, f** Chromatographic peaks of the accumulation of apigenin and apigenin-7-O-β-d-glucoside detected in susceptible and resistant pepper cultivars were calibrated against HPLC analytical standards. *, $P \leq 0.05$; **, $P \leq 0.01$; ns, no significant difference (paired *t* test)

Further, we evaluated the effects of apigenin, apigenin-7-O-β-d-glucoside, and their combination on the vegetative growth of economically important phytopathogenic fungi and oomycetes, including *P. sojae*, *Pyricularia*

oryzae, *C. siamense*, and *F. graminearum*. Exogenous application of each compound showed significant inhibitory efficacy across all species (Additional file 1: Figures S2–S5). Interestingly, unlike *P. capsici*, there was no

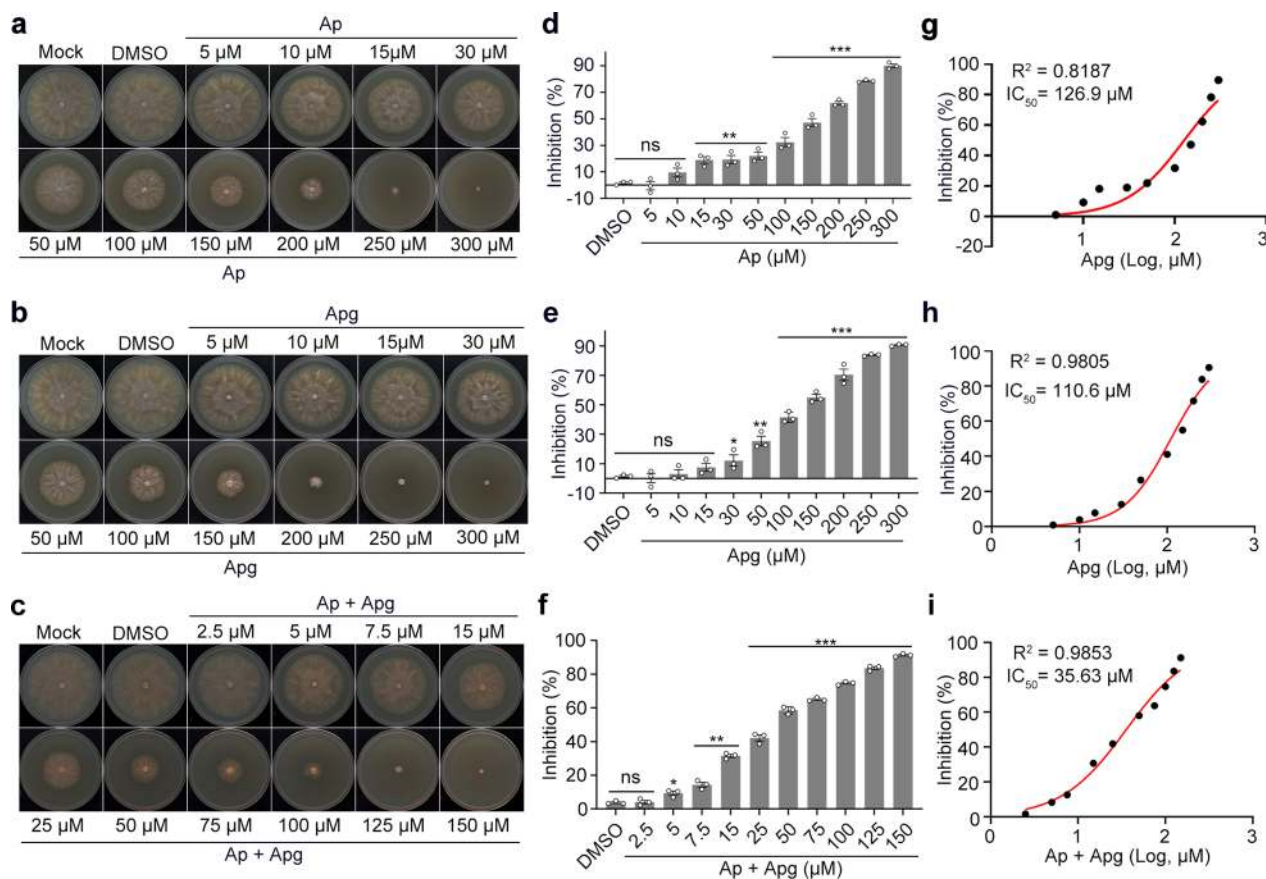


Fig. 2 The inhibitory effects of apigenins on the vegetative growth of *P. capsici*. **a–c** Plate assay of the inhibitory efficacy of various concentrations of apigenin, apigenin-7-O- β -D-glucoside, or their combination against the vegetative growth of *P. capsici*. **d–f** Statistical analysis of the data from **a–c**. **g–i** Determination of the concentrations (IC₅₀) corresponding to 50% inhibition in the vegetative growth of *P. capsici*. Ap: apigenin; Apg: apigenin-7-O- β -D-glucoside. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns: no significant difference (one-way ANOVA followed by Dunnett's multiple-comparison test)

substantial difference in the concentrations required to cause 50% inhibition in the vegetative growth of *P. sojae*, *Pyricularia oryzae*, *C. siamense*, and *F. graminearum*. The IC₅₀ values for *P. capsici* were 2–3 times lower than for these other species (Fig. 2 g–i, Additional file 1: Figures S2, S5g–i).

Exogenous apigenins trigger apoptotic-like collapse in vegetative hyphae of *P. capsici*

To gain further insight into the effects of apigenin, apigenin-7-O- β -D-glucoside, and their combination on the morphology and cellular dynamics of vegetative hyphae in *P. capsici*, LT1534 strains were cultured on TA agar media supplemented with exogenous apigenin (126.9 μ M), apigenin-7-O- β -D-glucoside (110.6 μ M), a cocktail of apigenin and apigenin-7-O- β -D-glucoside (35.63 μ M), DMSO (0.2% v/v), and the mock control group, then incubated under optimal growth conditions for 24 h. SEM analyses revealed that treatment

with apigenin and apigenin-7-O- β -D-glucoside induced significant collapse of hyphal cells, compared to DMSO and the control group (Fig. 3a). TEM analysis further demonstrated dynamic changes, including increased liposome formation, nuclear alterations, mitochondrial disintegration, vacuolar enlargement, and abnormal plasma membrane and cell wall morphology in hyphal cells treated with apigenin (Fig. 3b). Additional flow-cytometry analysis revealed a significant up-regulations in the induction of early apoptosis in response to exogenous apigenin (Ap 126.9 μ M), apigenin-7-O- β -D-glucoside (Apg 110.6 μ M), a cocktail of apigenin and apigenin-7-O- β -D-glucoside (Ap + Apg 35.63 μ M) (Fig. 3c, d), suggesting that the accumulation of glycosylated and non-glycosylated apigenin disrupts vital cellular processes and triggers apoptotic cell death, thereby interfering with the pathophysiological development of *P. capsici*.

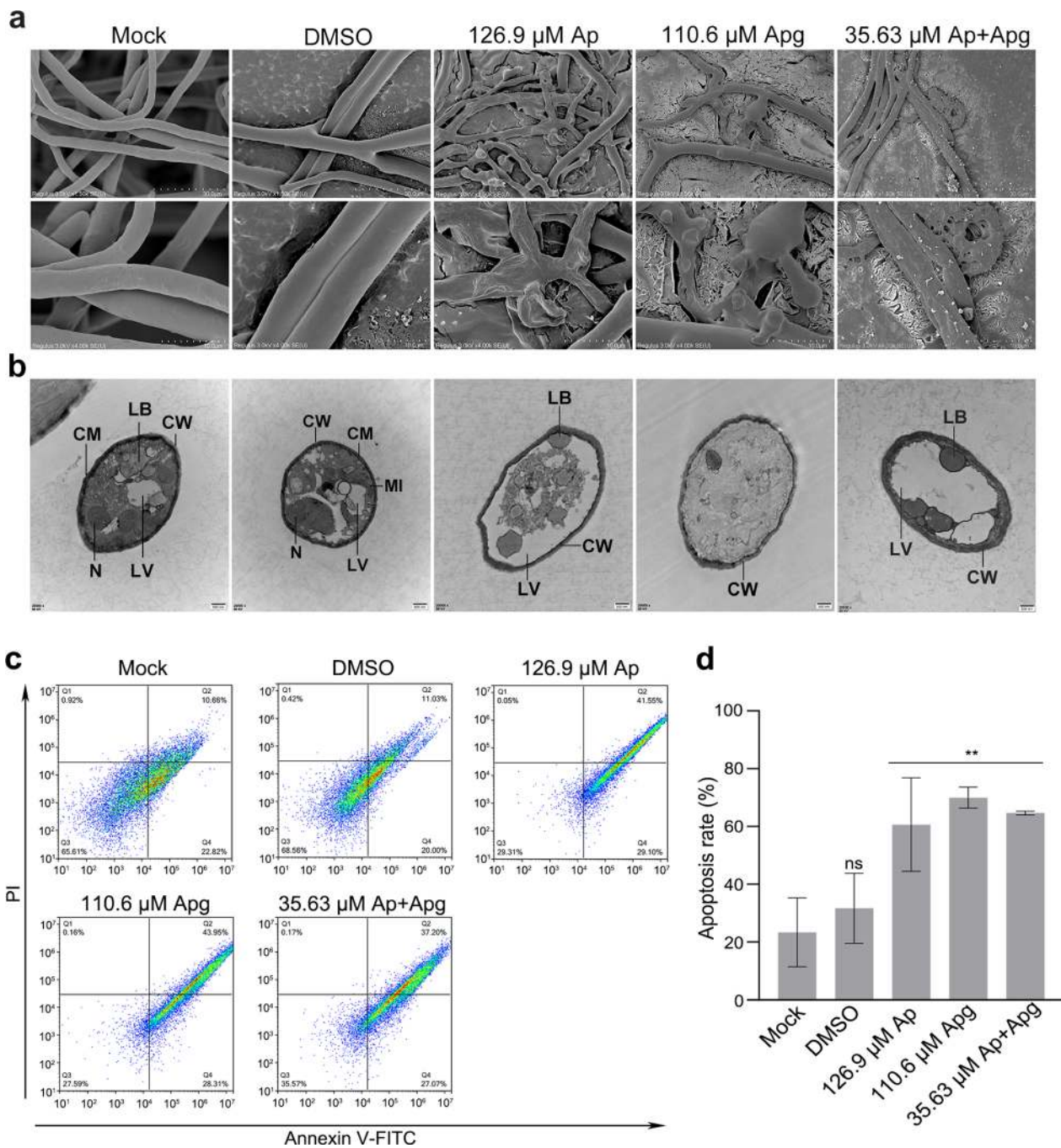


Fig. 3 Impacts of apigenin treatments on cellular integrity and induction of apoptosis in *P. capsici*. **a, b** Effects of various concentrations of apigenin (126.9 μ M Ap), apigenin-7-O- β -d-glucoside (110.6 μ M Apg), or a combination of both (35.63 μ M Ap + Apg) on the mycelium and cellular structure of *P. capsici*. Scale bars: 30 μ m (**a**-upper panel); 10 μ m (**a**-lower panel); 500 nm (**b**). **c** Flowcytometry dot plots showed the *P. capsici* LT1534's protoplasts treated with DMSO (0.2%, V_{vol}), 126.9 μ M Ap, 110.6 μ M Apg, and 35.63 μ M Ap + Apg. Q1: death induced by mechanical or instrument injury, Q2: late-stage apoptosis, Q3: living or intact cells, and Q4: early-stage apoptosis. **d** Statistical representation of flowcytometry-assisted evaluation of the influence of Ap, Apg, and Ap + Apg on the induction of apoptosis in *P. capsici*. CW: cellular wall; CM: cellular membrane; LV: lytic vacuoles; LB: lipid bodies; MI: mitochondrion; N: cellular nuclei. ** indicates significant difference between the treatment and the mock control (one-way ANOVA, parametric $P \leq 0.01$); ns: no significant difference; error bars indicate standard deviation (SD) of data from three independent biological replicates and three technical replicates

Apigenins significantly suppress asexual reproduction in *P. capsici*

Sporangia play a crucial role in the overwintering, reproduction, and dissemination of *P. capsici*. To investigate the influence of apigenin, apigenin-7-O- β -d-glucoside, or a cocktail of both on sporangium production in *P. capsici*, we assessed the sporangium production of LT1534 strains grown on TA agar medium supplemented with various concentrations of these compounds. Treatments included apigenin, apigenin-7-O- β -d-glucoside, a cocktail of both compounds, DMSO (0.2% v/v), and a mock control group. The results showed that apigenin and apigenin-7-O- β -d-glucoside, at 100 μ M (Fig. 4), and the cocktail at 25 μ M (Additional file 1: Figure S6), significantly reduced the sporangium production capabilities

of *P. capsici*. We concluded that the accumulation of apigenin and apigenin-7-O- β -d-glucoside in host tissues during pepper-*P. capsici* interaction also directly compromises the ability of *P. capsici* to reproduce, thereby limiting its pathogenesis.

Apigenins inhibit zoospore germination and suppress zoospore germination in *P. capsici*

To investigate the effects of apigenin, apigenin-7-O- β -d-glucoside, and their combination on zoospore release in *P. capsici*, we used the IC₅₀ value from the growth experiment, along with two concentrations (IC₅₀ $\pm$ 10 μ M) of each compound, to prepare sporangium suspensions. A 30 μ L sporangium suspension was inoculated on hydrophobic coverslips, placed in a moist sample box, and

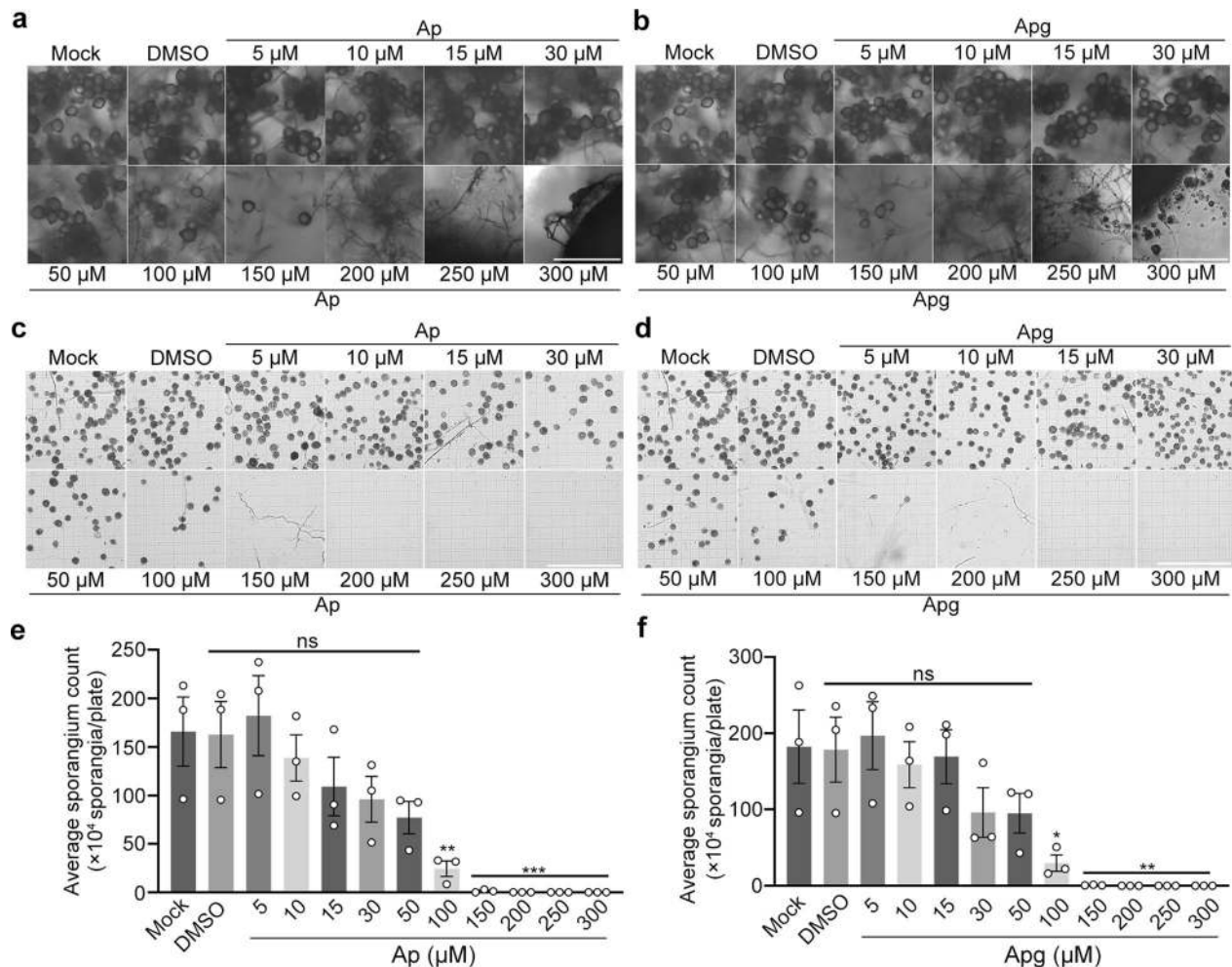


Fig. 4 Influence of apigenin and apigenin-7-O- β -d-glucoside on sporangium production in *P. capsici*. **a–d** Effects of different concentrations of apigenin (Ap) and apigenin-7-O- β -d-glucoside (Apg) on sporangium production. **e, f** Statistical analysis (one-way ANOVA, parametric analysis) of the data obtained from **c, d**. Scale bars: 50 μ m (**a, b**); 100 μ m (**c, d**). Asterisks indicate significant difference between the treatment and the mock control (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$); ns: no significant difference. The error bars indicate the standard deviation of data from three independent biological replicates and three technical replicates

incubated at 4°C for 30 and 60 min. Microscopy examinations revealed that, compared to DMSO (0.2% v/v) and the mock control, treatments with apigenin, apigenin-7-O- β -d-glucoside, or their combination significantly suppressed the rate of zoospore release in *P. capsici* (Fig. 5a–c). Additionally, the same treatments also led to a substantial reduction in zoospore germination efficiency, with the cocktail of apigenin and apigenin-7-O- β -d-glucoside exerting the most significant inhibitory effect (Fig. 5d–f).

Exogenous apigenin and apigenin-7-O- β -d-glucoside enhance resistance in susceptible pepper cultivars against *P. capsici*

To investigate the effects of apigenins on the disease caused by *P. capsici*, we pre-treated susceptible pepper seedlings with apigenin, apigenin-7-O- β -d-glucoside, and cocktails of apigenin and apigenin-7-O- β -d-glucoside, and incubated the treated seedlings in the dark for 30 min before inoculation with sporangia suspensions (3×10^5 sporangia/mL) of LT1534 strains. Results showed

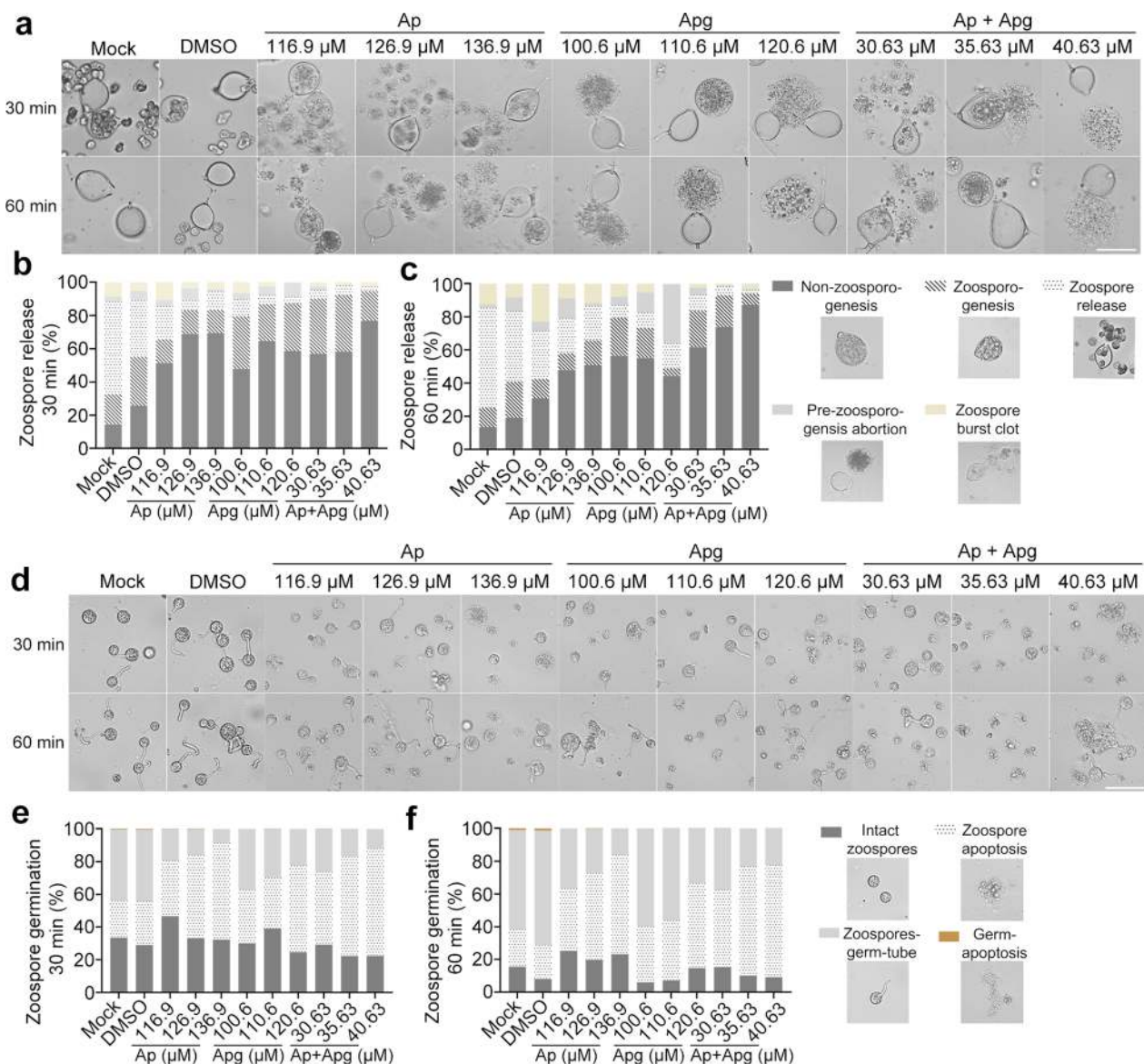


Fig. 5 Effects of apigenins on the formation, release, survival, and germination of zoospores in *P. capsici*. **a** The impact of various concentrations of apigenin (Ap), apigenin-7-O- β -d-glucoside (Apg), or their combination (Ap + Apg) on zoospore release and survival. **b, c** Bar graphs depict the statistical effects of these compounds on zoospore release and survival. **d** Influence of Ap, Apg, or Ap + Apg on the germination and survival of zoospores. **e, f** The bar graphs present the statistical evaluation of the effects on zoospore germination and survival. Scale bars in **a** and **d**: 20 μ m

that pre-treatment with apigenin ($\geq 126.9 \mu\text{M}$), apigenin-7-O- β -d-glucoside ($\geq 110.6 \mu\text{M}$), or a cocktail of apigenin and apigenin-7-O- β -d-glucoside ($\geq 35.63 \mu\text{M}$) significantly suppressed disease development (Fig. 6a, b).

Moreover, we observed no adverse effects on the pepper seedlings treated with apigenin, apigenin-7-O- β -d-glucoside, the cocktail, and the DMSO (0.2% v/v) (Fig. 6c). To further investigate the potential role of apigenins in disease resistance, we examined the effect of pre-treatment of the HNUCB0226 pepper seedlings with apigenins on the expression of a panel of selected pathogenesis-related (PR) genes encoding pathogenesis-related protein 1 (CaPR1), basic endochitinase B (CaCHT), ent-kaurene oxidase (CaKOL), ent-kaurene synthase (CaKSL7), and phenylalanine ammonia-lyase (CaPAL) (Bin et al. 2012). Quantitative RT-PCR analysis revealed an up-regulation in transcript levels of *CaCHT*, *CaKOL*, and *CaKSL7*, especially in response to apigenin and the cocktail treatments (Fig. 6d). Interestingly, the *CaKOL*, *CaKSL7*, and *CaPAL* showed relatively higher expression

at 5 dpi in pepper seedlings pre-treated with cocktail preparation of apigenin and apigenin-7-O- β -d-glucoside (Fig. 6e). Based on these results, we infer that the accumulation of apigenin and apigenin-7-O- β -d-glucoside may directly enhance pepper immunity against *P. capsici*.

Discussion

Apigenin, a naturally occurring plant flavonoid, exists in both non-glycosylated (aglycone) and glycosylated (glycone) forms (Lefort and Blay 2013; Tang et al. 2017). The biosynthesis of both forms of apigenin has been reported in numerous plants, including parsley, celery, and chamomile, inter alia (Tang et al. 2017). However, there are limited reports on the generation and accumulation of apigenin in pepper (Castillejo et al. 2022; Islam et al. 2023). Here, we demonstrated the generation and accumulation of apigenin and apigenin-7-O- β -d-glucoside in the leaf metabolome of three pepper cultivars. These observations suggest that the regulatory pathways and enzymes involved in apigenin biosynthesis

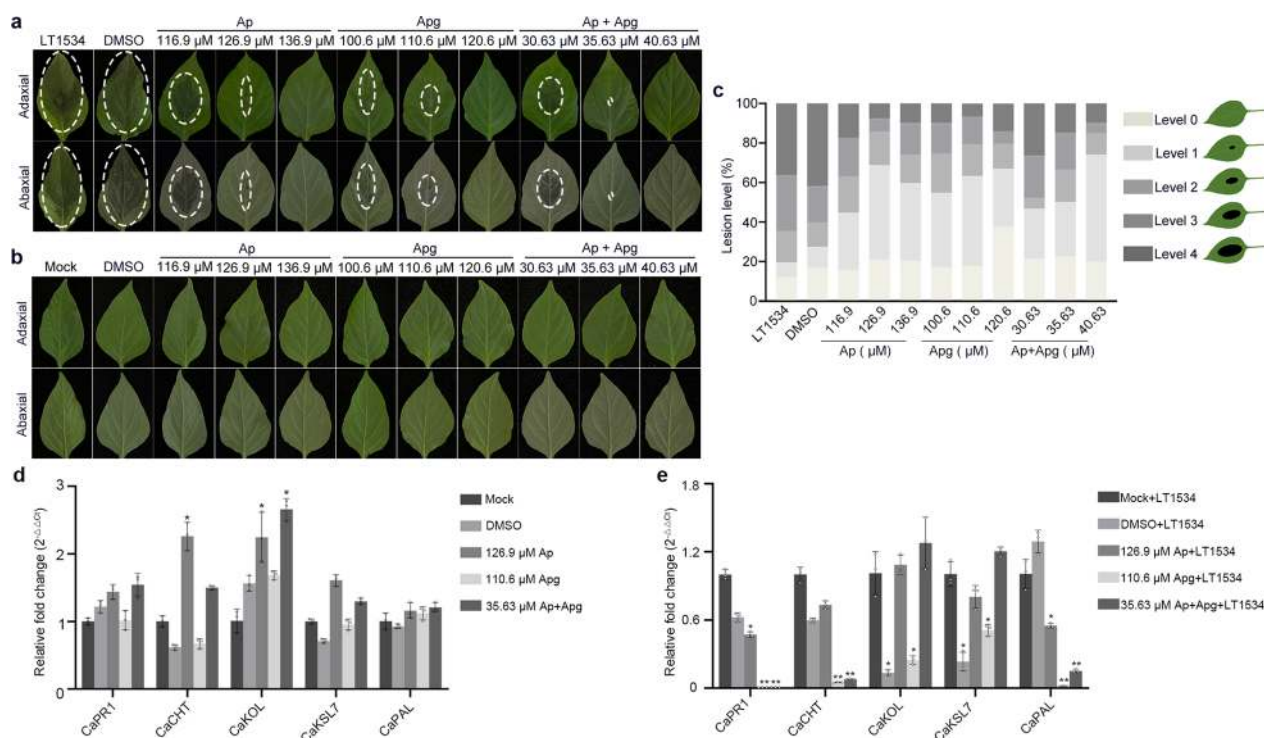


Fig. 6 Apigenin enhances resistance in susceptible pepper cultivars to *P. capsici*. **a** Exogenous application of apigenin (Ap), apigenin-7-O- β -d-glucoside (Apg), and their combination (Ap + Apg) significantly suppressed the development of Phytophthora blight disease. **b** Effects of different concentrations of Ap, Apg, or Ap + Apg on pepper leaves. **c** Effects of Ap, Apg, or Ap + Apg treatment on lesion development. **d** Effects of Ap, Apg, or Ap + Apg treatment on the expression of selected pathogenesis-related genes in *Capsicum annuum* without pathogen inoculation. **e** Bar graph showing the expression levels of pathogenicity-related genes at 5 dpi in *Capsicum annuum* seedlings pretreated (60 min before inoculation with *P. capsici*) with Ap, Apg, or Ap + Apg, 0.2% (v/v) DMSO (solvent control), and ddH₂O (mock). qPCR analysis was conducted using data from three independent biological experiments, each comprising three technical replicates. Asterisks indicate a significant difference between the treatment and the mock control (one-way ANOVA, parametric analysis, *, $P \leq 0.05$; **, $P \leq 0.01$). The error bars indicate the standard deviation of data from three independent biological replicates and three technical replicates

and glycosylation, including flavone synthases (FNS) (Righini et al. 2019; Tian et al. 2022; Luo et al. 2023), chalcone isomerase (CHI), chalcone synthase (CHS), 4-coumarate ligase (4-CL), cinnamate-4-hydroxylase (C4H) (Cheng et al. 2018), phenylalanine ammonia-lyase (PAL), UDP-glucose-7-O-glucosyltransferase (UGT) (Marín et al. 2017; Hou et al. 2023), and other cytochrome P450 enzymes (Peng et al. 2017), are relatively conserved in plants.

Moreover, apigenin levels in the leaf metabolome of the three pepper cultivars increased in response to *P. capsici* infection, underscoring its role in boosting plant immunity against pathogens. This finding is supported by previous research indicating that the biosynthesis and accumulation of apigenin enhance plant immunity against biotic stress by activating multiple plant defense mechanisms, including the generation of defense hormones such as salicylic acid and jasmonic acid and the expression of genes encoding PR proteins (Falcone Ferreyra et al. 2015). Flavonoid-mediated signaling events have also implicated in activating the biosynthesis of additional plant defense-associated metabolites, including terpenoids and diterpenoids (Sugimoto et al. 2022). Here, transcriptomic analysis revealed significant upregulation of genes encoding for PR proteins associated with flavonoid and diterpenoid biosynthesis pathways, including phenylalanine ammonia-lyase, ent-kaurene oxidase, and ent-kaurene synthase (Saito et al. 2013; Yamamura et al. 2018). These observations partly support the conclusion that apigenin and apigenin-7-O- β -d-glucoside directly or indirectly enhance plant immunity possibly by coordinating defense-related signaling cross-talk between metabolite biosynthetic pathways in *C. annuum*.

Plant flavonoids, including apigenin, generally possess antimicrobial activity against pathogenic bacteria and fungi, either by directly inhibiting the growth of pathogenic microbes within or around host tissues or indirectly by providing cellular cues that trigger the generation of defense-related compounds and hormones (Nayaka et al. 2014). Previous studies confirmed the inhibitory effects of apigenin against diverse human pathogenic bacteria, including *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Proteus mirabilis*, and *Klebsiella pneumoniae* (Nayaka et al. 2014). We demonstrated that the exogenous application of apigenin, apigenin-7-O- β -d-glucoside, or their combination significantly inhibited the vegetative morphology of economically significant oomycete and fungal species, including *P. capsici*, *P. sojae*, *Pyricularia oryzae*, *C. siamense*, and *F. graminearum*. These observations are consistent with previous findings regarding the significant inhibitory activities of non-glycosylated apigenin against sporangium formation of *P. capsici* (Kato et al. 2022) and a wide range of soybean

pathogens, including *Colletotrichum truncatum*, *Macrophomina phaseolina*, *Phoma exigua*, *P. sojae*, *Pythium ultimum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* (Jiang et al. 2012). These observations indicated that both non-glycosylated and glycosylated apigenin exhibit broad antimicrobial effects against pathogenic bacteria, oomycetes, and fungi. This partly supports the reasoning that differences in the levels of apigenin and apigenin-7-O- β -d-glucoside among pepper cultivars during *P. capsici* infection possibly account for the variations in resistance among the three pepper cultivars against *P. capsici*.

We also demonstrated that apigenin and apigenin-7-O- β -d-glucoside suppress the development of disease by interfering with multiple key stages in the life cycle of *P. capsici*. Specifically, not only do these compounds inhibit vegetative hyphal growth and reduce sporangium production, but they also inhibit zoosporogenesis, suppress zoospore release and germination of zoospores, and possibly impair encystment of sporangia (sporangia failing to undergo the zoosporogenesis process required for zoospore formation). Accordingly, we reasoned that blocking these stages effectively prevents infection before host penetration occurs. Thus, even though some free apigenin may accumulate in susceptible cultivars, the ability of the resistant cultivar to accumulate high levels of apigenin-7-O- β -d-glucoside, which likely acts synergistically with apigenin, appears critical for thwarting the pathogen at the early pre-invasion stage. This multi-step interference aligns with recent reports that apigenin treatments significantly reduce fungal and oomycete reproduction, including morphogenetic differentiation and spore production (Jiang et al. 2012), and partly supports the conclusions that the generation and accumulation of glycosylated and non-glycosylated in plants during pathogen invasion constitute a viable defensive strategy.

In *Candida albicans*, apigenin treatment triggered cell shrinkage and significantly compromised membrane integrity (Lee et al. 2018). Apigenin suppresses human carcinogenesis by accelerating apoptosis in cancer cells (Woo et al. 2020). Here, we showed that apigenin and apigenin-7-O- β -d-glucoside treatments caused hyphal cells in *P. capsici* to collapse, triggered the formation of lytic vacuoles, aggregation of lipid bodies, disintegration of cellular organelles, including nuclear and mitochondrial, and compromised cell wall and membrane integrity. Additionally, we demonstrated that apigenin or apigenin-7-O- β -d-glucoside, alone or in combination, significantly compromised zoospore survival in *P. capsici*. Accordingly, we speculate that the accumulation of glycosylated and non-glycosylated apigenin in plants disrupts disease development by inducing cell death in the invading pathogen. Future studies should investigate the

direct influence of apigenin on the initiation of the programmed cell death, including apoptosis or autophagy, in phytopathogenic oomycetes and fungi.

Apigenin acts as a buffer by regulating the generation and accumulation of cytotoxic compounds, including reactive nitrogen species (RNS) and reactive oxygen species (ROS). For instance, SH-SY5Y neuroblastoma cell lines treated with apigenin were protected against the cytotoxic effects of reactive radicals (Kim et al. 2021). Unlike neuroblastoma cells, treating malignant mesothelioma cell lines with apigenin modulates ROS generation and compromises mitochondrial integrity (Lee et al. 2020). Furthermore, we showed that pre-treatment of *P. capsici*-susceptible pepper cultivars with apigenin, apigenin-7-O- β -D-glucoside, or both enhances the resistance of pepper seedlings to *P. capsici* without causing physical or cellular injury in the pre-treated plants. These observations, coupled with previous research findings that implicated apigenin in promoting growth and stress tolerance in rice seedlings (Mekawy et al. 2018), suggest that apigenin may modulate different molecular targets in plants than in humans, bacteria, or fungi.

In conclusion, these findings provide a solid framework for screening protein targets of glycosylated and non-glycosylated apigenin in phytopathogenic oomycetes and fungi, advancing the development of sustainable apigenin-based strategies for controlling *P. capsici* and other important fungal pathogens.

Conclusions

This study demonstrated that intracellular levels of apigenin and apigenin-7-O- β -D-glucoside correlate with varying levels of resistance among three pepper cultivars to *P. capsici*. The results suggest that the production of these compounds in response to *P. capsici* infection significantly inhibits disease progression by suppressing the reproduction, pathogenic differentiation, and morphological development of *P. capsici*. Additionally, apigenin and apigenin-7-O- β -D-glucoside also inhibit a wide range of phytopathogenic oomycetes and fungi, including *P. sojae*, *Pyricularia oryzae*, *C. siamense*, and *F. graminearum*. These findings indicate that these compounds could serve as valuable natural agents for sustainable control of oomycete and fungal diseases, opening new opportunities for integrated disease management and the development of pepper cultivars with enhanced resistance.

Methods

Test materials and reagents

Apigenin (HPLC $\geq$ 98% purity) was purchased from Hebei Co-create Biotechnology Co., Ltd., Apigenin-7-O- β -D-glucoside (HPLC $\geq$ 98% purity) from Nanjing

Chunqiu Biological Engineering Co., Ltd. and DMSO from Biofroxx, Germany. Cell viability, apoptosis, and death were assessed using the Annexin V-FITC/PI Apoptosis Detection Kit (CX006L; Shanghai Epizyme Biomedical Technology Co., Ltd.).

Test strains and pepper cultivars

The wild-type strains of *Phytophthora capsici* LT1534, *Pyricularia oryzae* Guy11, *Phytophthora sojae* P6497, *Colletotrichum siamense*, and *Fusarium graminearum* PH-1 were obtained from the strain library of Tropical Plant Disease Control, Microbial Genomics, and Microbial Resource Utilization Laboratory at the Tropical Agriculture and Forestry College of Hainan University. Pepper cultivars HNUCB0081 (highly susceptible) and HNUCB0226 (moderately resistant) were provided by Professor Zhiwei Wang of the Tropical Agriculture and Forestry College of Hainan University. The completely resistant cultivar, XIAO ZHOU PI, was purchased from the Chunhua Seed Industry Center in Yichun City, Jiangxi Province, China.

Cultivation and preparation of samples from pepper seedlings for non-targeted metabolomics analysis

Leaf tissues of the individual pepper seedlings were spray-inoculated with sporangia suspension (3×10^8 spores/mL) and incubated at 25°C. Inoculated leaf tissues were collected at 1–12, 24–48, and 56–72 h post-inoculation (hpi) to capture early, mid, and late stages of infection. LT1534 strains cultured in V8 medium for 4 days were filtered through four layers of cheesecloth, rinsed with sterilized ddH₂O, and excess water was drained with autoclaved Whatman filter paper. The inoculated and non-inoculated leaf tissues and LT1534 samples were separately ground into fine powder in liquid nitrogen. Fifty milligrams of the ground samples were weighed into a 1.5 mL Eppendorf tube, and 800 μ L of extraction solvent (methanol:water=7:3 v/v, pre-cooled to -20°C) was added to the samples. An aliquot of 20 μ L internal standard was added to the content, and two small steel balls were added. The tubes were grouped and subjected to further grinding in a weaving grinder (50 Hz, 5 min). The homogenized samples were further ultrasonicated in a water bath at 4°C for 10 min and centrifuged at 25,000 g for 15 min at 4°C. An aliquot of 600 μ L of the supernatant was pipetted into 96-well plates. The filter plates were first rinsed with 200 μ L of 70% methanol, and then 500 μ L of the supernatant was filtered into clean glass vials. Finally, 50 μ L was used for non-targeted metabolomics analysis. The whole-metabolome of the inoculated and non-inoculated pepper seedlings was analyzed using 2777C UPLC (Waters, UK) liquid chromatography and Xevo G2-XS QTOF system (Waters, UK) mass

spectrometry instruments (BGI-Tech metabolomics platform in Shenzhen). Samples for non-targeted metabolomic profiling were obtained from six independent biological infection experiments with each consisting of five technical replicates.

Preparation of samples from pepper seedlings for targeted metabolomic analysis

Leaves of 6-week-old susceptible pepper cultivar (HNUCB0081), moderately resistant pepper cultivar (HNUCB0226), and highly resistant pepper cultivar (XIAO ZHOU PI) were inoculated with sporangium suspensions at a concentration of approximately 5×10^5 sporangia/mL. Forty microliters of the sporangium suspension were pre-incubated in the dark at 25°C for 24 h and then exposed to natural light for 24 h, along with a mock control group treated with autoclaved double-deionized water. The leaves were collected, ground into a powder with liquid nitrogen, and lyophilized for 48 h. Each sample (30 mg) was mixed with 1 mL of 70% methanol, vortexed at room temperature at 100% power for 20 min, and centrifuged at 9660 g at 4°C for 10 min. The resulting supernatants were pipetted into sterilized centrifuge tubes. One hundred microliters of supernatant were diluted with 900 µL of 70% methanol, vortexed, centrifuged, and 100 µL was used for quantitative metabolic analysis using Waters ACQUITY UPLC-XEVO TQ-S MS triple quadrupole liquid mass spectrometers at the Metabolomics Research Center, Straits Joint Research Institute, Fujian Agriculture and Forestry University.

Plate assays of the inhibitory effects of apigenin and its glucoside derivatives on the vegetative growth of oomycetes and fungi

Plugs of 4 mm in diameter containing freshly growing vegetative mycelia of the tested strains were used to inoculate agar plates supplemented with varying concentrations of apigenin, apigenin-7-O-β-d-glucoside, or a 1:1 mixture of both compounds. Tomato juice agar (TA) (10% tomato juice, 0.14% CaCO₃, 1.5% Agar) and V8 agar (10% V8 juice, 0.14% CaCO₃, 1.5% Agar) were used for *P. capsici* (LT1534) and *P. sojae* (P6497), respectively. Inoculated plates were incubated in the dark at 25°C for 4 days (*P. capsici*) and 10 days (*P. sojae*). For *Pyricularia oryzae* (Guy11), a complete medium (CM, 6 g yeast extract, 6 g casein hydrolysate, 10 g sucrose, 20 g agar) was used, and for *C. siamense* and *F. graminearum*, the potato dextrose agar (PDA, 200 g peeled potato chunks, 20 g D-glucose, 20 g agar) was used. The inoculated plates were incubated at 28°C for 10 days (*Pyricularia oryzae*), 7 days (*C. siamense*), and 4 days (*F. graminearum*). The inhibitory effects of apigenin, apigenin-7-O-β-d-glucoside, and their combination on the vegetative growth of *P. capsici*,

P. sojae, *Pyricularia oryzae*, *C. siamense*, and *F. graminearum* were evaluated by measuring colony diameters on their respective culture media.

Flow cytometry-assisted analysis of apoptosis in *P. capsici*

The annexin-V coupled with propidium iodide (PI) staining assay (Lakshmanan and Batra 2013) was employed to evaluate the apoptosis induction in *P. capsici*. The LT1534 strain was cultured in NPB medium (1.0 g K₂HPO₄, 3.0 g KNO₃, 0.5 g MgSO₄, 0.1 g CaCl₂, 2.0 g CaCO₃, 5.0 g D-sorbitol, 5.0 g D-mannitol, 5.0 g D-glucose, 2.0 g yeast extract, 2 mL vitamin stock, 2 mL trace elements) (Wang et al. 2018) for 24 h and then treated with 0.2% (v/v) DMSO (solvent control), apigenin (126.9 µM, Ap), apigenin-7-O-β-d-glucoside (110.6 µM, Apg), or a cocktail of apigenin and apigenin-7-O-β-d-glucoside (35.63 µM, Ap + Apg at a 1:1 molar ratio). Treated cultures were incubated for an additional 12 h.

Protoplasts were isolated from treated and mock control groups using a previously described protocol (Wang et al. 2018). The isolated protoplasts were resuspended in 200 µL binding buffer, after which 5 µL annexin V-FITC and 10 µL PI were added. Samples were incubated on ice in the dark for 10 min. Flow cytometric analysis was conducted using a high-sensitivity flow cytometer (Novo-Cyte Quantec VBYSR, Agilent, Singapore) to determine the proportion of apoptotic cells in apigenin-treated strains compared with the control groups.

Assays of sporangium formation, zoosporogenesis, and zoospore release in *P. capsici*

The LT1534 strain was cultured in TA medium supplemented with the tested compounds. The treated strains were incubated at 28°C in darkness for 4 days. The plates were further incubated under continuous light for 1–2 days to maximize sporulation. The sporangia were washed from the plates with sterile water, and the final volume of the sporangium suspension was adjusted to 1 mL. The suspension was mixed thoroughly, and 10 µL was placed on a hemocytometer and counted under a light microscope.

For assays of spore zoosporogenesis and zoospore release, the sporangia were washed from the plates supplemented with the tested compounds and filtered with sterilized Miracloth to generate sporangium suspensions with a concentration of 1×10^5 sporangia/mL. A 20 µL suspension was inoculated onto hydrophobic coverslips, kept in a moist chamber, and incubated at 4°C for 30 or 60 min. Microscopic assessment of zoosporogenesis, zoospore release, and survival in response to treatments was performed at 30 and 60 min post-inoculation (mpi) using a light microscope.

For the zoospore germination assay, sporangia were washed from culture plates with sterilized water and incubated at 4°C for 30 min to induce zoosporogenesis and zoospore release. The zoospores were filtered through two layers of Miracloth to obtain zoospore suspensions. The concentration of zoospores in suspensions was adjusted to 1×10^5 zoospores/mL. Zoospores in suspension were treated with the tested compounds, and 20 μ L of the suspension was inoculated on hydrophobic coverslips, placed in moist sample boxes, and incubated at room temperature. The effects of treatments on the germination efficiency of zoospores were monitored at 30 and 60 min under a light microscope.

Assays to evaluate the effects of apigenin on the virulence of *P. capsici*

Six-week-old pepper seedlings, at the 5–8 leaf stage and susceptible to *P. capsici*, were sprayed with the tested compounds and incubated in the dark for 30 min. The treated leaves were inoculated with sporangium suspensions (3×10^5 sporangia/mL). For each inoculation, 20 μ L of sporangia suspension was applied to the treated and untreated control leaves. The inoculated seedlings were incubated in a dark growth chamber at 27°C with 80%–95% humidity for 24 h and later transferred to a growth chamber with 12 h light–dark cycles. Disease severity was assessed by measuring lesion diameters on infected leaves.

Scanning electron microscopy and transmission electron microscopy investigation

P. capsici LT1534 was inoculated on a TA agar medium supplemented with tested compounds. After incubating in the dark at 25°C for 1 day, a mycelium sample was collected, frozen in liquid nitrogen for 10 min, and coated with a gold–palladium layer. Images were captured using a Regulus 8100 (HITACHI) scanning electron microscope. For transmission electron microscopy (TEM), the mycelium sample was fixed in 2.5% glutaraldehyde for 1 h, rinsed thrice with 0.1 M PBS (phosphate buffer saline) for 15 min each, and subsequently fixed in 0.1 M osmium tetroxide for 1–2 h. The sample was rinsed with 0.1 M PBS four times for 15 min each and dehydrated in a graded ethanol series (50%, 70%, 80%, 90%, 95%, 100%) for 15 min at each step. The sample was dehydrated with acetone (2–3 times, 15 min each) before embedding and staining with uranyl acetate and lead citrate. Images were acquired using an H-7650 (HITACHI) transmission electron microscope at the Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou, China.

Plant growth conditions and sample preparation for total RNA extraction and qPCR analysis

To investigate the expression profiles of protein-coding genes associated with disease progression in pepper plants, seedlings of the moderately resistant cultivar HNUCB0226 were grown under controlled laboratory conditions. The plants were cultivated in sterilized organic soil at 20°C–30°C and under 50%–75% relative humidity for approximately 6 weeks. Two experimental groups were established. In the first group, 6-week-old HNUCB0226 seedlings were independently sprayed with apigenin (126.9 μ M), apigenin-7-O- β -d-glucoside (110.6 μ M), a cocktail of apigenin and apigenin-7-O- β -d-glucoside (35.64 μ M), 0.2% (v/v) DMSO (solvent control), or double-distilled water (ddH₂O; mock control). The treated plants were incubated for 60 min, after which leaves from each treatment were harvested, immediately frozen in liquid nitrogen, and stored at –80°C.

In the second group, pepper seedlings were inoculated with sporangial suspensions prepared from *P. capsici* (LT1534) strains. The concentration of the sporangial suspension was adjusted to 2.0×10^4 sporangia/mL and used to spray-inoculate the seedlings. Inoculated leaves were collected 5 days post-inoculation (dpi), immediately frozen in liquid nitrogen, and stored at –80°C until further analysis.

For total RNA extraction, frozen leaf samples were ground to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. Total RNA was extracted following the manufacturer's protocol for the Trelief® RNAprep Pure Plant Plus Kit (for polysaccharide and polyphenolic-rich samples; Qingke, Beijing, China). Reverse transcription was performed using the SynScript® III RT SuperMix for qPCR Kit (Qingke, Beijing, China) to synthesize cDNA from the isolated RNA. The resulting cDNA samples were diluted threefold and used as templates for quantitative PCR (qPCR). qPCR assays were performed using the ArtiCanCEO SYBR qPCR Mix system (Additional file 2: Table S1) with gene-specific primers (Additional file 2: Table S1) on a StepOne Real-Time PCR System (StepOne/StepOnePlus, Applied Biosystems, USA). Actin (Gene ID: 107850541) was used as the internal reference gene. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Shabbir et al. 2022).

Statistical analyses

All statistical analyses were performed using data from at least three biological experiments, each with 3–5 technical replicates. Data were computed using GraphPad Prism 9.5.1 software. Paired t-tests were performed for pairwise comparisons, and non-parametric tests

(e.g., Kruskal–Wallis) were used for group comparisons when assumptions for parametric ANOVA were not met. Statistical significance was denoted as follows: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Abbreviations

Ap	Apigenin
Apg	Apigenin-7-O- β -d-glucoside
CM	Complete media
CHI	Chalcone isomerase
CHS	Chalcone synthase
C4H	Cinnamate-4-hydroxylase
CW	Cellular wall
CM	Cellular membrane
DMSO	Dimethyl sulfoxide
dpi	Day post-inoculation
FNS	Flavone synthases
hpi	Hour post-inoculation
HPLC	High-performance liquid chromatography
LV	Lytic vacuoles
LB	Lipid bodies
mpi	Min post-inoculation
MI	Mitochondrion
N	Cellular nuclei
PBS	Phosphate buffer saline
PR	Pathogenesis-related
PDA	Potato dextrose agar
PAL	Phenylalanine ammonia-lyase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TA	Tomato-juice agar
UGT	UDP-glucose-7-O-glucosyltransferase
UPLC	Ultra performance liquid chromatography
4-CL	4-Coumarate ligase

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42483-026-00414-z>.

Additional file1: Figure S1. The exclusive generation of apigenin and apigenin-7-O- β -d-glucoside in pepper seedlings inoculated with *P. capsici*. **Figure S2.** Influence of different concentrations of apigenins on the vegetative growth of *P. sojae*. **Figure S3.** Apigenin treatments significantly inhibited the vegetative development of *P. oryzae*. **Figure S4.** Apigenins suppress the morphological development of *C. siamense*. **Figure S5.** Effects of apigenin and apigenin-7-O- β -d-glucoside, alone and combined, on the vegetative growth of *F. graminearum*. **Figure S6.** Effects of the combination of apigenin and apigenin-7-O- β -d-glucoside on sporangium production of *P. capsici*.

Additional file2: Table S1. List of primers used in this qPCR.

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Author contributions

JN, JB, LL (Linying Li), and JR conceived and designed the experiment. JN, JB, and BL sourced funding for the research. JN, LL (Linying Li), JR, BL, XQ, RS and KZ performed the experiments. JN, JB, LL (Lili Lin), MS, and WL conducted data and method validation. LL (Linying Li), JR, BL, and XQ carried out visualization. LL (Linying Li), JR, BL, XQ, WL, and LL (Lili Lin) drafted the manuscript, while JN, JB, MS, and LL (Lili Lin) revised the same. All authors contributed to the final manuscript.

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Data availability

The data supporting the findings of this study are available and can be obtained from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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