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Expression Pattern of Some Salicylic Acid-Responsive Defense Genes during the *Helianthus annuus* L. - *Orobanche cumana* Wallr. Interaction

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ABSTRACT

Sunflower (*Helianthus annuus* L.) production is severely affected by the root holoparasite *Orobanche cumana* Wallr., making the development of resistant genotypes a priority for sustainable crop protection. This study examined the expression patterns of salicylic acid (SA)-responsive genes *PR5, HaDef1,* and *HaAC1* in resistant (Favorit) and susceptible (Performer) sunflower genotypes during pre- and post-attachment stages of infection. The results revealed clear genotype-dependent differences. The resistant genotype exhibited early induction of *PR5* and *HaDef1* prior to parasite establishment and sustained upregulation of these genes during later developmental stages of the parasite. In contrast, the susceptible genotype showed weak, transient, or repressed expression of these defense-related genes. *HaAC1* displayed a more limited and stage-specific contribution, particularly during post-attachment phases. Overall, resistance to *O. cumana* is associated with timely activation and maintenance of SA-dependent defense responses, whereas susceptibility correlates with insufficient transcriptional activation. These findings highlight the importance of genotype-specific regulation of SA-responsive genes in sunflower resistance to broomrape.

Keywords: sunflower, *Orobanche cumana*, pathogenesis-related genes, gene expression, post-attachment, pre-attachment.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops worldwide, but its production is severely constrained by broomrape (*Orobanche cumana* Wallr.), an obligate root holoparasite capable of causing substantial yield losses, particularly in heavily infested areas. The parasite attaches to host roots, establishes vascular connections, and withdraws water and nutrients, leading to reduced growth, seed yield, and, in severe cases, complete crop failure (Rîşnoveanu et al., 2016). The development of resistant genotypes represents the most effective and environmentally sustainable strategy for broomrape control. In this context, a deeper understanding of the molecular mechanisms underlying host resistance is essential for the successful breeding and selection of tolerant sunflower cultivars.

In Eastern Europe, substantial contributions to the understanding of sunflower - *Orobanche cumana* interactions have been made by Maria Păcureanu-Joiţa and collaborators. Their studies have demonstrated the continuous evolution of broomrape populations and the emergence of new, highly virulent races capable of overcoming previously effective resistance genes (Păcureanu-Joița et al., 2002, 2012). Furthermore, research conducted under Romanian conditions highlighted the dynamic nature of host-parasite interactions and the need for continuous identification of new genetic sources of resistance, including those derived from wild sunflower species and breeding lines (Anton et al., 2016). These findings emphasize that resistance to *O. cumana* is not static, but rather a complex and evolving trait requiring integrative physiological and molecular approaches for its proper characterization.

Plants have evolved sophisticated defence systems to counteract biotic stress, among which systemic acquired resistance (SAR) represents a key whole-plant defence

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mechanism. It is triggered following a localized hypersensitive response, often accompanied by pathogen-induced tissue necrosis, and is mediated through the salicylic acid (SA) signaling pathway (Yasuda et al., 2023).

This response involves the activation of stress-responsive genes and the accumulation of pathogenesis-related (PR) proteins, which play a central role in plant immune responses. PR proteins function either by degrading pathogen components or by facilitating hormone signaling to activate defence pathways (Van Loon and Van Kammen, 1970). Among these, chitinases, glucanases, ribonucleases, hydrolases, defensins, and peroxidases are widely recognized as key components of plant immunity, exhibiting diverse enzymatic activities and antimicrobial effects against a broad spectrum of pathogens, including fungi, bacteria, viruses, and insects. (Van Loon and Van Kammen, 1970; Han and Schneiter, 2024).

Among PR families, *PR1*, *PR2*, and *PR5* encoding genes are widely used as molecular markers of SA-mediated systemic acquired resistance (Ali et al., 2018; Fang et al., 2025). The PR5 family comprises thaumatin-like proteins (TLPs), which are structurally related to thaumatin, a sweet-tasting protein from *Thaumatococcus daniellii*. (Van der Wel and Loeve, 1972). TLPs are well known for their antimicrobial properties, primarily through interactions with microbial plasma membranes, often resulting in membrane permeabilization or osmotic imbalance (Jiao et al., 2018). These proteins exhibit multiple biological functions, including glucan binding, putative endo-β-1,3-glucanase-related activity (Sakamoto et al., 2006), xylanase inhibition (Fierens et al., 2007), and plasma membrane permeabilization (Vigers et al., 1991), which collectively contribute to their broad antifungal activity (Sharma et al., 2021; Islam et al., 2023). TLPs are synthesized in response to both pathogen attack and environmental stress, and their expression is associated with resistance traits.

In sunflower, *PR5*-1 shares sequence similarity with antifungal PR5 members, including osmotin and thaumatin (Hu et al., 2003).

Defensins, small cysteine-rich peptides, are also key SA-responsive defence components. They are constitutively expressed in storage and reproductive tissues or induced upon pathogen attack, mechanical injury, or systemic immune responses (Vriens et al., 2014). Plant defensins inhibit fungal growth, partly by impairing hyphal elongation (Broekaert et al., 1995; Hu et al., 2003).

Several defensins have been identified in sunflower, including the anther-specific SF18-like protein (Domon and Steinmetz, 1994) and *HaDef1* (Hu et al., 2003). *HaDef1* is strongly induced in resistant sunflower roots upon infection by the root parasitic plant *Orobanche cumana*, suggesting its role in parasite necrosis and defence (Letousey et al., 2007).

Another SA-responsive gene, *HaAC1*, encodes an aldo-keto reductase and has been identified as a molecular marker of the SA signaling pathway in sunflower. Aldo-keto reductases contribute to plant resistance to both biotic and abiotic stress and play roles in secondary metabolism, redox balance, and plant-microbe interactions (Letousey et al., 2007; Sengupta et al., 2015).

The aim of this study is to investigate the expression dynamics of some key salicylic acid-responsive pathogenesis-related genes (*PR5*, *HaDef1* and *HaAC1*) in sunflower (*Helianthus annuus*) during infection by the root holoparasite *Orobanche cumana* in both resistant (incompatible) and susceptible (compatible) interactions, across early (pre-attachment) and late (post-attachment) infection phases covering key developmental stages.

MATERIAL AND METHODS

Plant material and growth conditions

Experiments were conducted on root tissue collected from sunflower (*Helianthus annuus* L.) seedlings of two hybrids: Favorit, which carries resistance genes against *Orobanche cumana*, and Performer, without resistance genes. Seeds were provided by NARDI Fundulea, Romania.

Pre-Attachment Phase

Seeds were germinated and grown until the two-true-leaf stage in Petri dishes containing a perlite substrate. To induce biotic stress, *O. cumana* seeds, pre-germinated on exudate from a susceptible genotype, were introduced into the plant growth medium. Root tissue samples were collected at four post-inoculation time points: 2, 6, 12, and 24 hours.

Post-Attachment Phase

For post-attachment experiments, sunflower plants were grown in 5 L pots in a 1:1 soil:sand mixture to facilitate root system handling, with three pots per treatment and 6-10 seeds per pot (three biological replicates). Artificial infestation of the substrate with *O. cumana* seeds (not preconditioned) was carried out at a rate of 37 mg of seeds per 200 g of soil mixture. Plants grown in non-infested substrate served as controls. Root tissue samples were collected dynamically over 67 days, corresponding to four pathogen developmental stages: formation of the first attachments (18-21 days), tubercle development (35 days), formation of underground shoots (53 days), and development of aerial shoots (67 days).

Total RNA Isolation and cDNA Synthesis

Total RNA was extracted from root tissue samples using TRI reagent (Applied Biosystems) following the standard protocol. RNA quality and quantity were assessed via spectrophotometry (260 and 280 nm) and 1% agarose gel electrophoresis. cDNA synthesis was performed using RevertAid RT reagent with Oligo$(dT)_{18}$ primers and random hexamers according to the manufacturer's instructions (Fermentas). The experimental design included three biological replicates for each experimental variant.

Gene Expression Analysis by Real-Time PCR

Relative expression levels of target genes (Table 1) were determined using real-time PCR on a QuantStudio® 5 system (Applied Biosystems) with the following amplification program: 95°C for 10 min; 5 cycles of 95°C for 15 s and 64°C for 20 s; followed by 40 cycles of 95°C for 15 s and 60°C for 40 s. The reaction mixture contained Maxima SYBR Green/ROX PCR Master Mix reagent (Fermentas), gene-specific primers (0.4 µM), and 2 µl of cDNA.

Table 1. Genes and primers used in the study

Gene	Gene Bank ID	Primer sequence		Amplicon length, bp
		Forward	Revers	
PR5	AF364864.1	tgcagccgtgttcactattc	catatacgggctcctgctgt	138
HaAC1	AF030301.1	ggccccaaaaccaacgaaat	ttcacaagcagccctaacgt	147
HaDef	AF364865.1	atggccaaaatttcagttgc	ctcccaagacttgcactggt	168
Actin	AF282624.1	gctaacagggaaaagatgactc	actggcataaagagaaagcacg	96

All reactions were carried out in triplicate, including non-template controls. Relative gene expression was quantified using the cycle threshold (Ct) method. Normalized expression levels were calculated as $2^{-\Delta Ct}$, where $\triangle Ct = (Ct_{target\ gene} - Ct_{reference\ gene})$. To compare gene expression between samples (plants grown in the absence of broomrape seeds as control and in the presence of the parasite), fold change was determined using the $2^{-\Delta\Delta Ct}$. When $2^{-\Delta\Delta Ct}<1$, downregulation was expressed as the negative inverse value ($-1/2^{-\Delta\Delta Ct}$), representing the fold reduction in gene expression (Schmittgen and Livak, 2008).

Expression differences, expressed as fold change (FC), were considered biologically relevant for FC $\geq$ 1.5 and statistically significant at $p \leq 0.05$. The Student's t-test of significance was applied. Data shown in figures represent mean values ± standard deviation.

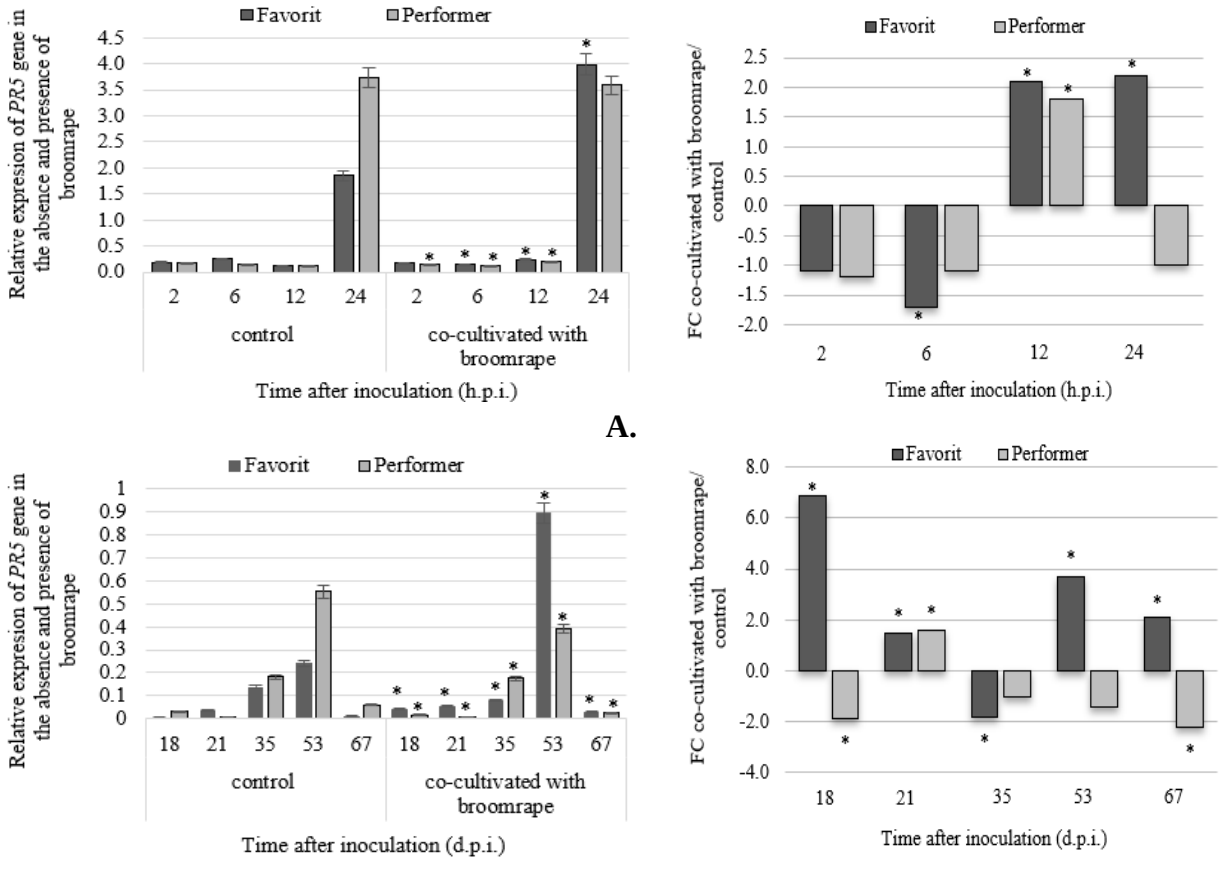
RESULTS AND DISCUSSION

The expression dynamics of three salicylic acid (SA)-associated genes (*PR5, HaDef1*,

and *HaAC1*) were analyzed in broomrape-resistant (Favorit) and susceptible (Performer) sunflower genotypes. Gene expression was measured at different time points covering both pre-attachment and post-attachment phases of the host-parasite interaction, in the presence and absence of broomrape.

It is known that *PR5* is a key marker gene of the salicylic acid signaling pathway and plays an important role in systemic acquired resistance (SAR), contributing to the activation of defence responses that restrict pathogen spread in infected and distal tissues. Its involvement in pathogen defence has been revealed in various plant species, as for example, in sunflower infected with *Sclerotinia sclerotiorum*, *PR5*-1 transcript accumulation at 24 hpi was significantly higher in partially resistant lines than in susceptible ones (Monazzah et al., 2018). Also, a strong induction of *PR5*-1 was observed in *Brassica napus* resistant genotypes following inoculation with the same pathogen (Wang et al., 2012). Similarly, differential expression of *PR5* genes has been associated with resistance to *Fusarium proliferatum* in garlic, further supporting the role of *PR5* genes in salicylic acid-mediated defence across diverse plant-pathogen interactions (Anisimova et al., 2021).

According to the results of our research *PR5* expression increased progressively in both the absence and presence of broomrape (Figure 1), reaching a maximum at 24 hours post-inoculation (hpi).



Note: An asterisk (*) denotes statistically significant differences ($p \leq 0.05$, t-test) compared to a control group

Figure 1. Relative expression levels and fold change of the *PR5* gene during the pre-attachment (A) and post-attachment (B) stages of the *Helianthus annuus* L. - *Orobanche cumana* Wallr. pathosystem

During the early stages of the host-parasite interaction, *PR5* transcript accumulation was moderately induced in the roots of both genotypes at 12 hpi (Favorit: 2.1-fold change; Performer: 1.8-fold change), similarly to the response observed by Letousey et al. (2007).

At 24 hpi, expression further increased in the resistant genotype Favorit (2.2-fold

change), indicating rapid activation of defence-related transcriptional responses. In contrast, the susceptible genotype Performer exhibited a decline in *PR5* expression comparative to uninfected control at 24 hpi.

During the post-attachment phase, *PR5* was significantly upregulated in resistant hybrid Favorit during all period of co-cultivation, with minor exceptions. The highest increasing under biotic stress conditions at 18 dpi (fold change 6.9) and 53 dpi (fold change 3.7), corresponding to parasite attachment and, respectively, to the underground shoot development phase. The expression peaks in both hybrids were established at 53 dpi, while, by comparison, sensible hybrid Performer exhibited especially significant repression at key developmental stages of the parasite (e.g. -1.9, -2.3 and -2.2 fold at 18, 53, respectively, 67 dpi).

These differences point to a stronger, more durable activation of SA-related defence in the resistant genotype across parasite development stages. According to Letousey et al. (2007), during the late post-attachment phase, the *PR5* gene was expressed only in the roots of the resistant genotype. Similarly, Sestacova et al. (2016) reported that at advanced stages of infection (90 days after sowing), *PR5* and *HaDef1* participate in the defensive response. In contrast, in *Arabidopsis thaliana* parasitized by *Orobanche ramosa*, SA-regulated genes (*PR-1, PR-2,* and *PR-5*) were not activated, as reported by Vieira Dos Santos et al. (2003). These findings highlight species- and genotype-dependent variation in SA-mediated defence responses to broomrape infection.

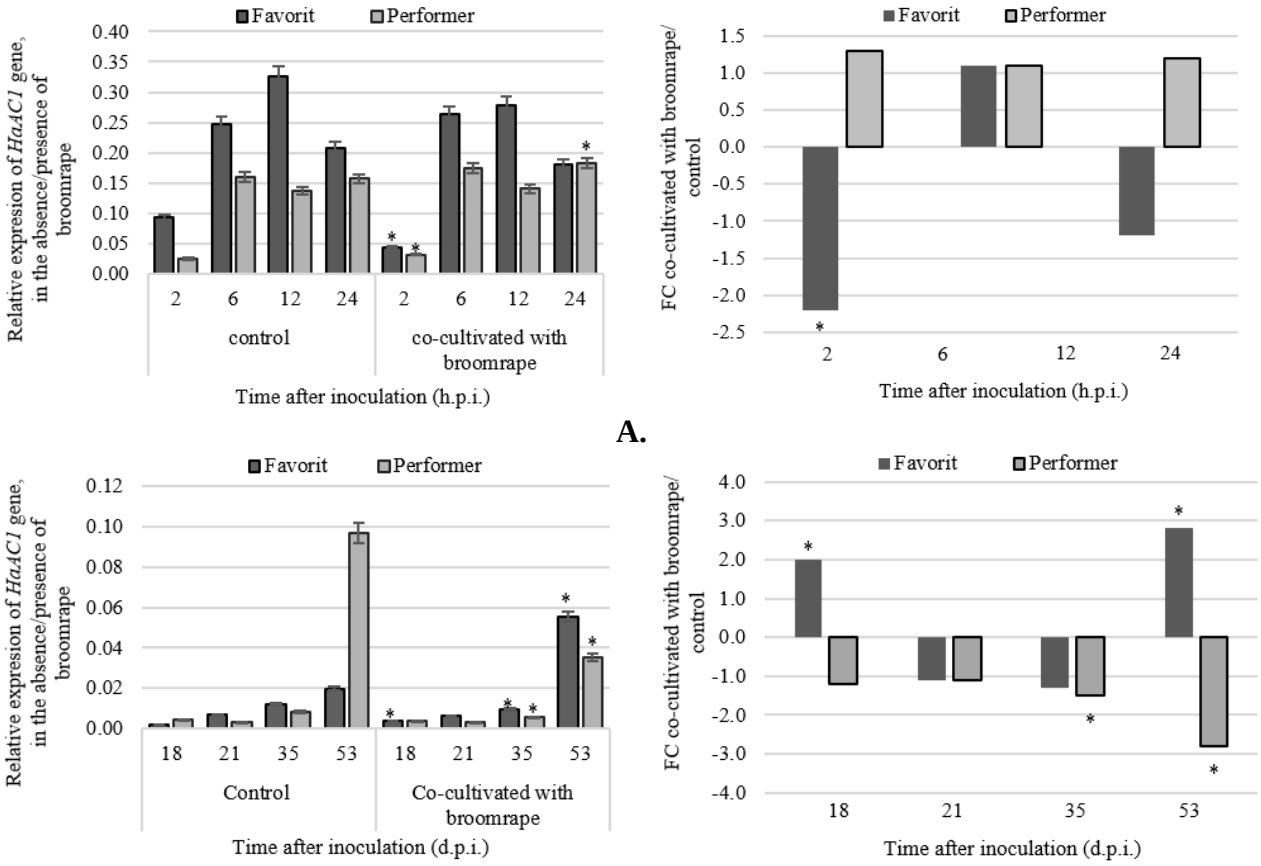
The *HaAC1* gene (Figure 2) analysed during the pre-attachment phase of host-parasite contact exhibited contrasting expression patterns between the two sunflower genotypes. In the resistant genotype Favorit, *HaAC1* transcript levels gradually increased, reaching a maximum after 12 hpi, followed by a decline in both experimental systems (cultivation of sunflower in the presence or in the absence of broomrape). Under infection background, changes in gene activity were mostly not significant, with the exception of a downregulation (fold change - 2.2) observed at 2 hpi.

In the Performer genotype, the transcriptional activity of *HaAC1* was about 2 times lower than in the resistant genotype, and no significant accumulation of transcripts was detected following co-cultivation with sunflower broomrape. These findings are partially consistent with those reported by Mazeyrat et al. (1998), who described distinct temporal patterns of *HaAC1* expression in compatible and incompatible *Helianthus annuus* - *Plasmopara halstedii* interactions. Thus, rapid and strong induction occurred in incompatible combinations within 6 h, peaking at 24-48 h, whereas in compatible interactions, *HaAC1* transcript accumulation was delayed until 48 h and was transient, becoming undetectable by 72 h (Radwan et al., 2005). Similarly, Letousey et al. (2007) reported that *HaAC1* expression was induced early in resistant sunflower genotypes, with amplification around 8 hpi, while no induction occurred in susceptible genotypes.

However, induction of *HaAC1* gene expression in the resistant genotype was detected during the post-attachment phase (18 and 53 dpi), with fold changes of 2.0 and 2.8, respectively, indicating its involvement in later-stage defence responses. In contrast, the susceptible genotype exhibited repression at later stages, with maximum downregulation observed at 35 and 53 dpi (-1.5- and - 2.8-fold, respectively). These results are in agreement with those obtained by Letousey et al. (2007), who revealed increased *HaAC1* expression in the roots of the sunflower genotype resistant to broomrape, with peak induction at 21 dpi.

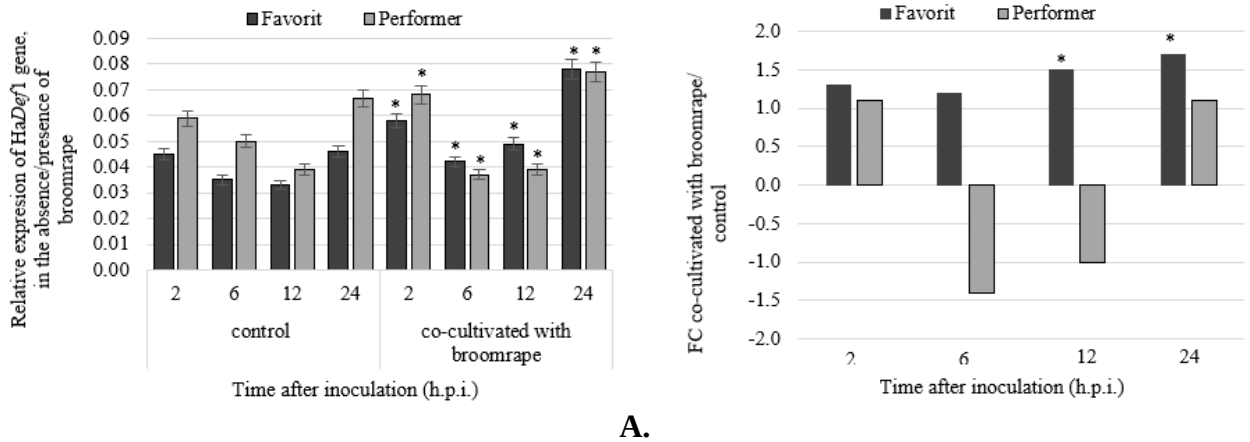
Contrary to the expression pattern observed for *HaAC1*, the *HaDef1* gene showed a different response during the early stages of interaction. The resistant genotype Favorit exhibited higher *HaDef1* transcript accumulation during co-cultivation with seeds of the parasitic weed broomrape, compared to the control (Figure 3). Similarly,

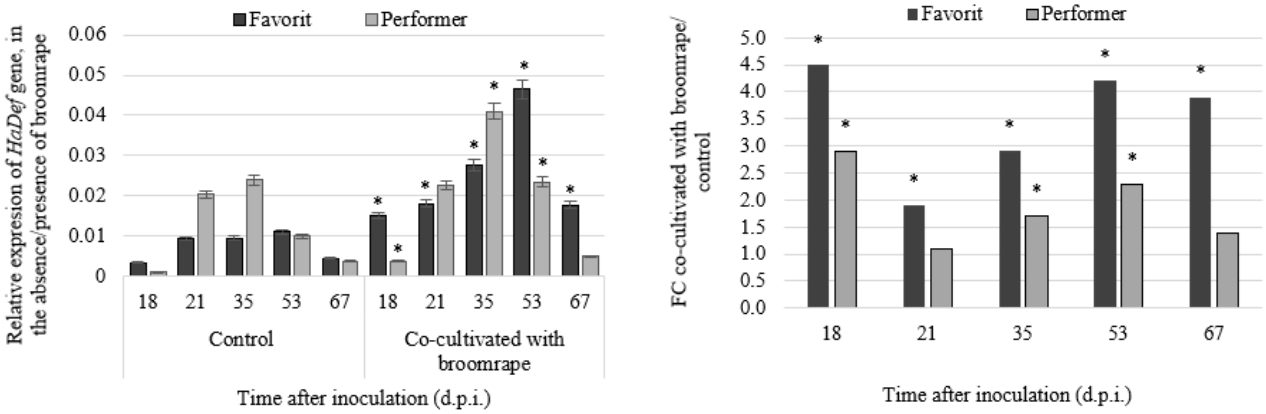
to the data reported by Letousey et al. (2007), *HaDef1* is upregulated especially in the roots of resistant sunflower genotype, with no major changes in susceptible hybrid. Thus, at 12 and 24 hpi, Favorit showed a significant increase, with fold changes of 1.5 and 1.7, respectively.



Note: An asterisk (*) denotes statistically significant differences ($p \leq 0.05$, t-test) compared to a control group

Figure 2. Relative expression levels and fold change of the *HaAC1* gene during the pre-attachment (A) and post-attachment (B) stages of the *Helianthus annuus* L. - *Orobanche cumana* Wallr. pathosystem





B.

Note: An asterisk (*) denotes statistically significant differences ($p \leq 0.05$, t-test) compared to a control group

Figure 3. Relative expression levels and fold change of the *HaDef1* gene during the pre-attachment (A) and post-attachment (B) stages of the *Helianthus annuus* L. - *Orobanche cumana* Wallr. pathosystem

During the post-attachment stages, *HaDef1* expression remained upregulated in Favorit, with fold changes ranging from 1.9 to 4.5, reaching a maximum at 18 dpi and 53 dpi (fold changes 4.5 and 4.2, respectively), consistent with enhanced resistance. In Performer, moderate upregulation was observed at some time points (fold change 2.9 at 18 dpi and 2.3 at 53 dpi) under the influence of the parasite. These patterns support the role of defensin as a component of inducible defence activated through the SA pathway, with a more robust response in the incompatible interaction.

Studies on host-parasitic plant systems using *Helianthus annuus* L. - *Orobanche cumana* Wallr. as a model, indicate that susceptible hosts often lack salicylic acid-associated activity, as was previously reported in the interactions between *Orobanche ramosa* and *Arabidopsis* (Vieira Dos Santos et al., 2003) and tomato with *Phelipanche ramosa* (Torres-Vera et al., 2016). In contrast, resistant cowpea responses to *Striga gesnerioides* are linked to stronger upregulation of the SA-responsive PR-5 transcript compared with susceptible interactions (Li et al., 2009).

CONCLUSIONS

The present study demonstrates that SA-associated genes (*PR5*, *HaAC1*, and *HaDef1*) show distinct, genotype-dependent expression patterns during the sunflower-broomrape interaction, reflecting their role in resistance. Defense responses were different between the pre-attachment and post-attachment stages.

During the pre-attachment stage, the resistant genotype Favorit exhibited a moderate early induction of *PR5* and *HaDef1* (at 12, 24 hpi), indicating rapid activation of SA-mediated signaling prior to parasite establishment. *HaAC1* showed only weak involvement at this stage. By contrast, the susceptible genotype Performer displayed weaker, reduced transcriptional activation, suggesting insufficient early defense priming.

In the resistant genotype Favorit, *PR5* and *HaDef1* maintained strong upregulation throughout key developmental phases of the parasite, whereas *HaAC1* was activated specifically at 18 and 53 dpi. By contrast, in the susceptible genotype Performer, *PR5* was repressed, and *HaDef1* showed modest induction, with transcript levels preferentially lower than those observed in Favorit. Generalizing, these findings indicate that effective resistance to broomrape is associated with both early (pre-attachment) activation and sustained (post-attachment) reinforcement of SA-dependent defense pathways, whereas susceptibility is linked to impaired or insufficient transcriptional responses across both stages of interaction.

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