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Stage-dependent variation in protease and α-amylase activities in *Rumex maritimus* L. infected by *Ustilago parletoreii* F.A. Waldheim

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Abstract
Fungal pathogens frequently reconfigure host primary metabolism, yet biochemical responses in many wild plant–smut systems remain uncharacterized. This study investigates stage-specific changes in protease and α-amylase activities in leaf and shoot tissues of *Rumex maritimus* naturally infected by *Ustilago parletoreii*. Protease activity in leaves increased from 0.038 µg/mg crude enzyme protein/min in controls to a peak of 0.049 µg/mg crude enzyme protein/min at pre-sporulation, while shoots rose from 0.013 to 0.028 µg/mg crude enzyme protein/min during sporulation, indicating intensified protein turnover associated with defense activation. A-Amylase activity displayed contrasting tissue dynamics: leaves increased from 0.162 to 0.185 mg glucose/mg protein/hr during early infection but sharply declined to 0.10 mg glucose/mg crude enzyme protein/hr at mature sporulation, whereas shoots exhibited a sustained rise from 0.310 to 0.405 mg glucose/mg crude enzyme protein/hr. These patterns reveal coordinated nitrogen and carbon remobilization, accompanied by tissue-specific metabolic exhaustion during advanced infection. Collectively, the findings provide the first biochemical evidence of enzymatic reprogramming in the *R. maritimus–U. parletoreii* interaction and highlight protease and α-amylase activities as sensitive indicators of infection progression.

Keywords: *Rumex maritimus*, *Ustilago parletoreii*, protease activity, α-amylase, smut infection

Introduction

Plant responses to fungal invasion require rapid and highly coordinated metabolic and proteolytic adjustments that reallocate carbon and nitrogen to support defense, repair, and survival (Bolton, 2009; La Camera *et al*., 2004) [3, 7]. Primary metabolism is therefore tightly integrated with immune responses: carbohydrate mobilization supplies energy and carbon skeletons for biosynthesis of defensive compounds, while proteolysis recycles nitrogen and generates signaling peptides that modulate immunity (Zeeman *et al*., 2010; van der Hoorn, 2008; Trouvelot *et al*., 2014) [14, 16, 20].

Proteases constitute a large and functionally diverse enzyme class in plants, performing central roles during pathogen challenges, ranging from the degradation of damaged proteins and pathogen targets to the generation of signaling peptides and the execution of programmed cell death (van der Hoorn & Jones, 2004; van der Hoorn, 2008) [16, 17, 18]. The MEROPS peptidase database documents the multiplicity of plant peptidases and underscores their biochemical and evolutionary diversity, which underpins diverse defensive functions (Rawlings *et al*., 2008; Rawlings *et al*., 2018) [11, 12].

Concomitantly, starch-degrading enzymes such as α-amylases and downstream maltose/glucose-producing pathways are frequently upregulated following biotic stress to provide soluble sugars for the energetic and biosynthetic demands of defense (Zeeman, Kossmann & Smith, 2010) [20]. Sugar fluxes can also act as signals that interact with phytohormone networks and influence resistance phenotypes (Trouvelot *et al*., 2014; Essmann *et al*., 2008) [5, 14]. Indeed, carbohydrate remobilization is a double-edged sword: while it fuels host defense, liberated sugars may also be exploited by pathogens (Bolton, 2009; Morkunas & Ratajczak, 2014) [3].

Smut fungi (Ustilaginales), particularly *Ustilago* spp., are biotrophic pathogens that provoke dramatic host reprogramming during colonization. The *Ustilago maydis*–maize system is a paradigmatic model demonstrating how a smut fungus manipulates host development and metabolism to favor its life cycle (Bölker, 2001; Brefort *et al*., 2009; Vollmeister *et al*., 2012) [2, 4, 19]. Ustilaginomycetes employ secreted effectors and enzymes that reconfigure host cellular processes, including carbohydrate metabolism and cell wall integrity, facilitating fungal proliferation and sporulation (Brefort *et al*., 2009; Lanver *et al*., 2017) [4, 8].

Empirical studies across diverse plant–pathogen systems report coordinated changes in hydrolases and proteases during infection. For example, hydrolase profiles—including amylases and proteases—shift in potato during *Phytophthora infestans* challenge, and protease induction has been observed in maize infected with *Fusarium* spp., reflecting conserved features of host metabolic remodelling (Tsvetkov *et al*., 2023; Naz *et al*., 2021) [10, 15]. Such work supports the concept that protein degradation and starch hydrolysis are core components of the host response to fungal attack (Rico *et al*., 2021; Bolton, 2009) [3, 13].

Despite this general understanding, host- and tissue-specific dynamics of protease and α-amylase activities remain underexplored in many wild plant species and non-model pathosystems. *Rumex maritimus* is an ecologically and physiologically distinct taxon for which biochemical responses to smut infection have not been well characterized; likewise, *Ustilago parletoreii* is a less-studied smut species relative to *U. maydis*. Determining stage-specific enzyme dynamics in leaves and shoots can therefore reveal tissue vulnerability, timing of resource remobilization, and potential biochemical markers of infection.

Given that protease- and amylase-mediated metabolic adjustments are central features of plant responses to fungal

pathogens (Rico *et al*., 2021; Tsvetkov *et al*., 2023) [13, 15], investigating these pathways in *R. maritimus* may reveal crucial insights into tissue susceptibility, infection progression, and pathogen-induced resource remobilization. Accordingly, this study examines stage-dependent variation in protease and α-amylase activities in leaf and shoot tissues of *R. maritimus* across five infection stages—pre-flowering, flowering, pre-sporulation, sporulation, and mature-sporulation—following natural infection by *U. parletoreii*. By integrating host enzymatic responses with established metabolic frameworks, this work provides the first biochemical characterization of this unique host–smut interaction.

Materials and Methods

Plant Material and Pathogen Inoculation

Healthy *Rumex maritimus* plants were collected from the field located at Maharabi, Imphal West, Manipur and maintained under controlled greenhouse conditions (25 ± 2 °C, 60–70 % relative humidity, 16 h light/8 h dark photoperiod). The fungal pathogen (chilling spore) *Ustilago parletoreii* was obtained from the Department of Plant Physiology, Manipur University.

For infection, leaves and shoots were inoculated with a freshly prepared spore suspension of *U. parletoreii* (1 × 10^6 spores/mL) using a sterile brush method. Control plants were treated with sterile distilled water. Inoculated plants were maintained under high humidity (90–95 %) for 48 h to facilitate infection and then returned to standard greenhouse conditions. Sampling was conducted across five developmental stages, comprising two control stages (pre-flowering and flowering) and three infection stages (pre-sporulation, sporulation, and mature sporulation).

Sample Collection

Leaf laminae and young shoots were collected from both control and infected plants at each developmental stage. Samples were stored in a deep freezer until enzyme extraction.

Enzyme Extraction

Plant tissues (~0.5 g) were ground in 5 ml of ice-cold extraction buffer (50 mM phosphate buffer, pH 5.4, containing 3 mM $CaCl_2$) using a chilled mortar and pestle. The homogenate was centrifuged at 2,000 rpm for 20 min at 4 °C. The supernatant was collected as a crude enzyme extract for protease and α-amylase assays.

Protease Activity Assay

Protease activity was determined using casein as substrate following the method of (Mahadevan and Sridhar, 1986) with minor modifications. 0.5 ml of enzyme extract was incubated with 0.5 ml of 1 % (w/v) casein solution in 50 mM phosphate buffer (pH 7.0) at 37 °C for 30 min. The reaction was stopped by adding 1 ml of 10 % (w/v) trichloroacetic acid. After centrifugation (2,000 × rpm, 20 min), the absorbance of the supernatant was measured at 650 nm. Protease activity was expressed as µg of tyrosine released per mg crude enzyme protein per minute (µg/mg protein/min).

α-Amylase Activity Assay

α-Amylase activity was measured according to the starch–iodine method (Hirasawa, 1989) [6]. Enzyme extract (0.5 ml) was incubated with 0.5 ml of 1 % soluble starch in 50 mM phosphate buffer (pH 5.4) at 37 °C for 30 min. The reaction was stopped by adding 1 ml of DNS reagent, boiled for 5 min, cooled, and the absorbance was recorded at 540 nm. α-Amylase activity was expressed as mg glucose released per mg crude enzyme protein per hour (mg glucose/mg protein/hr) using a glucose standard curve.

Protein Determination

Protein concentration in the crude enzyme extracts was determined by the Lowry method using bovine serum albumin as a standard.

Results and discussion

Protease activity

Protease activity in *Rumex maritimus* (Table 1) showed clear variation across infection stages. In leaves, activity decreased slightly from the control value of 0.038 µg/mg crude enzyme protein/min to 0.036 µg/mg crude enzyme protein/min at pre-flowering, then increased steadily during flowering (0.047 µg/mg crude enzyme protein/min) and pre-sporulation (0.049 µg/mg crude enzyme protein/min). A decline was observed at mature-sporulation (0.034 µg/mg crude enzyme protein/min). Shoots followed a similar pattern, rising from a control of 0.013 µg/mg crude enzyme protein/min to 0.016, 0.021, and 0.028 µg/mg crude enzyme protein/min through pre-flowering, flowering, and sporulation, before dropping to 0.021 µg/mg crude enzyme protein/min at mature-sporulation. These increases reflect pathogen-induced proteolysis, a known defense strategy where proteases remove damaged proteins and activate defense peptides (van der Hoorn & Jones, 2004; Rawlings *et al*., 2018) [11, 17, 18]. Similar protease upregulation has been reported in maize during *Fusarium* infection (Naz *et al*., 2021) [10]. The eventual decline aligns with tissue senescence under heavy pathogen load (López-Castillo *et al*., 2018).

Table 1: Determination of changes in protease activity in different stages of infection in the different tissues of *Rumex maritimus* infected by *Ustilago parletoreii*

Plant sample	Uninfected (control)		Infected with *Ustilago parletoreii*		
	Pre-flowering (µg/mg crude enzyme protein/min)	Flowering (µg/mg crude enzyme protein/min)	Pre-sporulation (µg/mg crude enzyme protein/min)	Sporulation (µg/mg crude enzyme protein/min)	Mature-sporulation (µg/mg crude enzyme protein/min)
Leaf	0.038	0.036	0.047	0.049	0.034
Shoot	0.013	0.016	0.021	0.028	0.021

α-Amylase activity

α-Amylase activity (Table 2) also exhibited tissue-specific shifts. Leaf activity increased from the control (0.162 mg glucose/mg protein/hr) to 0.174 and 0.185 mg glucose/mg crude enzyme protein/hr at pre-flowering and flowering. However, a marked decrease occurred during sporulation (0.113 mg glucose/mg crude enzyme protein/hr) and mature-sporulation (0.10 mg glucose/mg crude enzyme

protein/hr). Shoots maintained higher activity overall, rising from 0.310 mg glucose/mg crude enzyme protein/hr in controls to 0.312, 0.326, and 0.405 mg glucose/mg crude enzyme protein/hr through pre-flowering, flowering, and sporulation, with a slight reduction at mature-sporulation (0.385 mg glucose/mg crude enzyme protein/hr). This early activation indicates starch breakdown to supply sugars essential for stress responses (Zeeman *et al*., 2010) [20]. Similar trends have been reported in potato during *Phytophthora infestans* infection (Tsvetkov *et al*., 2023) [15]. The sharp decline in leaf amylase suggests depletion of carbohydrate reserves or tissue damage, while shoots maintain activity due to slower structural deterioration, a pattern observed in other host–fungus systems (Biemelt & Sonnewald, 2006) [1].

Together, the enzymatic profiles demonstrate that *U. parletoreii* triggers an integrated nitrogen–carbon remobilization program in *R. maritimus*, characterized by enhanced proteolysis and starch turnover. Such coordinated metabolic shifts represent a hallmark of plant responses to invasive fungal pathogens and are consistent with recent models of immune-associated metabolic rewiring (Rico *et al*., 2021) [13]. The magnitude and timing of these responses further suggest tissue-specific vulnerabilities, with leaves undergoing more rapid metabolic decline than shoots during advanced pathogen development.

Table 2: The variation in the a-amylase activity of the *Rumex maritimus* in the lamina and young shoot of different stages of *Rumex maritimus* infected by *Ustilago parletoreii*

Plant sample	Uninfected (control)		Infected with *Ustilago parletoreii*		
	Pre-flowering (mg glucose/mg crude enzyme protein/hr)	Flowering (mg glucose/mg crude enzyme protein/hr)	Pre-Sporulation (mg glucose/mg crude enzyme protein/hr)	Sporulation (mg glucose/mg crude enzyme protein/hr)	Mature-Sporulation (mg glucose/mg crude enzyme protein/hr)
Leaf	0.162	0.174	0.185	0.113	0.10
Shoot	0.310	0.312	0.326	0.405	0.385

Conclusion

Infection by *Ustilago parletoreii* induces strong metabolic restructuring in *Rumex maritimus*, characterized by elevated protease activity and early-stage stimulation of α-amylase. These shifts reflect intensified protein turnover and carbohydrate mobilization as core components of the host defense response. The sharp enzymatic declines at mature infection stages highlight progressive tissue deterioration and metabolic exhaustion. Overall, the findings show that coordinated nitrogen and carbon remobilization is central to the host's response and reveal clear tissue-specific susceptibility during fungal progression.

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Competing interests

The authors have declared that no competing interests exist.

Authors' Contributions

All the authors have given equal contributions. All the authors read and approved the final manuscript.

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