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Research article

Physiological and biochemical responses of *Rosa chinensis* Jacq. 'Old Blush' to waterlogging stress under a controlled greenhouse environment

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Abstract

Waterlogging stress significantly impairs the growth and physiological performance of many plant species. Using a completely randomized design, this study aimed to evaluate the effects of varying degrees of abiotic waterlogging stress on morphological and biochemical parameters in *Rosa chinensis* Jacq. 'Old Blush' under greenhouse conditions. Results indicate that the response of the Chinese rose to waterlogging varied with both the intensity and duration of the stress compared with the control group. Key quantitative results showed a progressive decline in leaf chlorophyll content, reaching a reduction of approximately 10% under severe, prolonged stress compared to the control. Concurrently, antioxidant enzyme activities (e.g., peroxidase and catalase) increased 2- to 3-fold, and leaf electrolyte leakage, an indicator of membrane damage, rose significantly ($P \leq 0.05$). Stomatal observations indicated a trend toward closure as stress duration increased, suggesting a restriction in gaseous exchange. Overall, the findings elucidate specific stress-response thresholds and provide a mechanistic framework linking stomatal behavior, pigment degradation, and oxidative stress in this cultivar. This work advances the fundamental understanding of waterlogging tolerance in roses and identifies potential trait targets for breeding more resilient ornamental plants in the face of climate variability.

Keywords: General Stress Response, *Rosa chinensis* Jacq., Rosaceae, Waterlogging.

Introduction

Ornamental plants have been valued by human civilizations for thousands of years, contributing not only to aesthetic appearance but also to environmental and psychological well-being. Among these, roses are particularly prized, often called the "queen of flowers" due to their diverse shapes, colors, and fragrances, making them important for both ornamental and commercial purposes (Ashrafi Kalteh *et al*., 2022).

Recent advances in genomics have significantly enhanced our understanding of rose biology. High-quality genomic sequences from derived haploid germplasm have provided new insights into key traits such as flowering time, floral development, self-incompatibility, and thorn density (Saint-Oyant *et al*., 2022; Saint-Oyant *et al*., 2018). These developments open new avenues for targeted breeding and genetic improvement.

Although extensive research has elucidated the biotic and abiotic stresses affecting roses, there is a notable gap in our understanding of their physiological and biochemical responses specifically to flooding stress, a growing environmental challenge linked to global climate change (Sarmast *et al*., 2024). Over recent decades, the increasing frequency and severity of flooding events have posed a substantial threat to agricultural productivity and plant health worldwide. Waterlogging, characterized

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by excess soil water, creates hypoxic or anoxic conditions around the roots due to restricted oxygen availability. These oxygen-deprived environments critically impair essential physiological processes, notably aerobic respiration, prompting plants to rely on less efficient anaerobic pathways such as fermentation. This metabolic shift affects energy production, carbohydrate metabolism, and overall plant energy, often resulting in undersized growth, developmental suspensions, and substantial yield damages (Manghwar *et al*., 2024).

In response to waterlogging, plants display a complex collection of morphological, physiological, and biochemical adaptations. Morphologically, plants may develop adventitious roots and aerenchyma tissues to enhance oxygen diffusion; physiologically, they alter hormonal levels, particularly ethylene and abscisic acid, to synchronize stress signaling; and biochemically, they activate specific pathways, including glycolysis and the tricarboxylic acid cycle, regulated by key genes and signaling networks. Of particular importance are the genes involved in anaerobic fermentation, reactive oxygen species (ROS) scavenging, and morphological modifications, all of which contribute to flood tolerance. At the molecular level, considerable progress has been made in decoding these responses. Moreover, flooding-induced oxidative stress, driven by ROS accumulation, further threatens cellular integrity, necessitating forceful antioxidant mechanisms (Manghwar *et al*., 2024; Ashraf, 2012).

While considerable research has advanced our understanding of plant responses to waterlogging in model species and crops, critical gaps persist in translating this knowledge to ornamental woody perennials. Specifically, for *Rosa chinensis*, the intricate interactions between signaling pathways and the genetic determinants of its tolerance remain largely unexplored. More importantly, key physiological and anatomical adaptations are still poorly characterized. It is unknown how *R. chinensis* coordinates its stomatal behavior—a primary gatekeeper for gas exchange and water loss—with underlying biochemical responses in mesophyll cells (e.g., antioxidant enzyme activity, chlorophyll degradation) under progressive waterlogging stress. Furthermore, the role of root system plasticity in this species during and after flooding has not been systematically investigated. These specific unknowns limit our ability to develop targeted strategies for enhancing resistance in roses. This study explicitly addresses these gaps by investigating the 'Old Blush' cultivar under controlled flooding conditions. The novelty of our work lies in the integrated, time-resolved analysis of three critical and underexplored aspects in roses: (1) dynamic stomatal conductance and aperture regulation in relation to stress duration, (2) concurrent biochemical responses (chlorophyll, carotenoids, antioxidant enzymes, proline content, and electrolyte leakage) in leaves, and (3) root morphological plasticity and viability post-stress. By correlating stomatal behavior with leaf-level biochemistry and root health, our research moves beyond merely documenting stress. It aims to establish causal links between early stomatal closure, photosynthetic decline, and oxidative damage, while evaluating root tolerance thresholds. This comprehensive approach provides a mechanistic framework that advances current knowledge beyond existing studies on roses or woody ornamentals, which often focus on a single parameter. The findings will be instrumental in identifying key traits for breeding or selecting waterlogging- resistant rose cultivars, thereby supporting sustainable ornamental horticulture in regions increasingly vulnerable to erratic rainfall and climate extremes.

Materials and Methods

Rosa chinensis Jacq. 'Old Blush' was kindly provided as a gift from Dr. Wang at Nanjing Agricultural University, China. Semi-hardwood cuttings, each approximately 15 cm in length, were collected from clonally propagated mother plants. These cuttings were maintained in a mist chamber to promote rooting, using a soil mixture composed of equal parts (by volume) of cocopeat and perlite. Following the emergence of adventitious roots, rooted cuttings were transplanted into 14-cm-diameter pots (1.2 Liters) filled with the aforementioned soil mixture and maintained under controlled greenhouse conditions. Fertigation was performed every 14 days using a half-strength Hoagland nutrient solution.

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Waterlogging treatment

The experiment was conducted in a standard greenhouse environment to control temperature, humidity, and photoperiod. Plants were grown under a 16/8 h (day/night) photoperiod using lights with an intensity of 50-100 µmol/ m^2/s^{-1}. The average temperature and relative humidity were 25.5 °C± 3 and 55% ± 5, respectively.

Uniform plants, matching in size and leaf number, were selected for the treatments. Waterlogging was applied for durations of 3, 6, 9, and 12 days. An additional subset of plants exposed to 12 days of waterlogging was allowed to recover for 4 days under normal greenhouse conditions to evaluate post-stress resistance. For waterlogging, each pot was carefully submerged in a larger plastic container, ensuring that the entire pot and the plant's crown were immersed in water, with only the stems and leaves remaining above the water surface. The water level was maintained consistently during the treatment periods. Data collection occurred at days 0 (before waterlogging), 3, 6, 9, and 12, as well as after the recovery period.

Measurement of morphological parameters

The fresh and dry weights of the aerial parts of each plant were recorded. Stem diameter was measured at a height of 5 cm above the crown using a digital caliper. Additionally, the weights of roots and shoots were determined separately to assess biomass allocation.

Electrolyte leakage test

To evaluate leaf membrane integrity, a 0.5 g leaf disc was excised from each plant and placed into a test tube containing 10 mL of distilled water. The samples were shaken gently at room temperature for 24 hours. The initial electrical conductivity (EC_1) of the solution was then measured using a calibrated digital EC meter. Subsequently, the samples were autoclaved at 121°C for 15 minutes to disrupt cell membranes, then cooled to room temperature, and the final electrical conductivity (EC_2) was recorded. The ion leakage rate was calculated as:

$$\boldsymbol{Electrolyte\ leakage} = \frac{\boldsymbol{EC1}}{\boldsymbol{EC2}} * \mathbf{100}$$

Proline and pigment content

Proline content in shoots was quantified following the method described by Bates *et al*. (1973). Briefly, homogeneous tissue samples were extracted with sulfosalicylic acid, reacted with acid-ninhydrin and glacial acetic acid, and the resulting complex was extracted with toluene. The absorbance of the toluene phase was measured at 520 nm using a spectrophotometer. Following the protocol of Hiscox and Israelstam (1979), chlorophyll and carotenoids were extracted from fresh leaf tissue using 80% acetone.

Enzymatic activity

Catalase (CAT) activity was measured as described by Chu *et al*. (2016). Briefly, 1 mL of reaction mixture containing 50 mM phosphate buffer (pH 7) and 15 mM H_2O_2 was mixed with 50 µL of enzyme extract. The decrease in absorbance at 240 nm was recorded over 1 minute.

Peroxidase (POX) activity was determined as described by Chance and Maehly (1995). A mixture consisting of 33 µL of enzyme extract, 1 mL of substrate solution containing 28 mM guaiacol, and 5 mM H_2O_2 in 50 mM phosphate buffer (pH 7) was prepared. Changes in absorbance at 470 nm were monitored every 10 seconds for one minute.

Stomatal morphological observation

Stomatal characteristics, including length, width, and aperture, were examined using an Olympus optical microscope (Olympus, Japan). Measurements were taken at six time points: days 0, 3, 6, 9, and 12, and after the recovery period. Photographs of the stomata were captured at 40× magnification (Shor *et al*. 2010). Stomatal impressions were obtained from the abaxial leaf surface using clear nail polish. For each treatment and time point, three leaves were sampled to evaluate stomatal parameters.

Statistical analysis

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Statistical analyses were performed using SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). The experiment was arranged in a completely randomized design (CRD) with six treatments and four replicates per treatment, for a total of 24 individual plants (one rooted cutting per pot). Means were compared using Duncan's multiple range test at a significance level of $P < 0.05$.

Results and discussion

In response to waterlogging stress, many of the *R. chinensis* developmental and physiological characteristics were severely affected. For example, total leaf number and lateral branch growth decreased significantly under flooding stress; as such, the number of axillary shoots on the main stem and the number of leaves were reduced to half after 12 days of waterlogging stress compared to control plants (Fig. 1A, B). The research results indicated that the mean root length was also influenced only after 12 days of waterlogging.

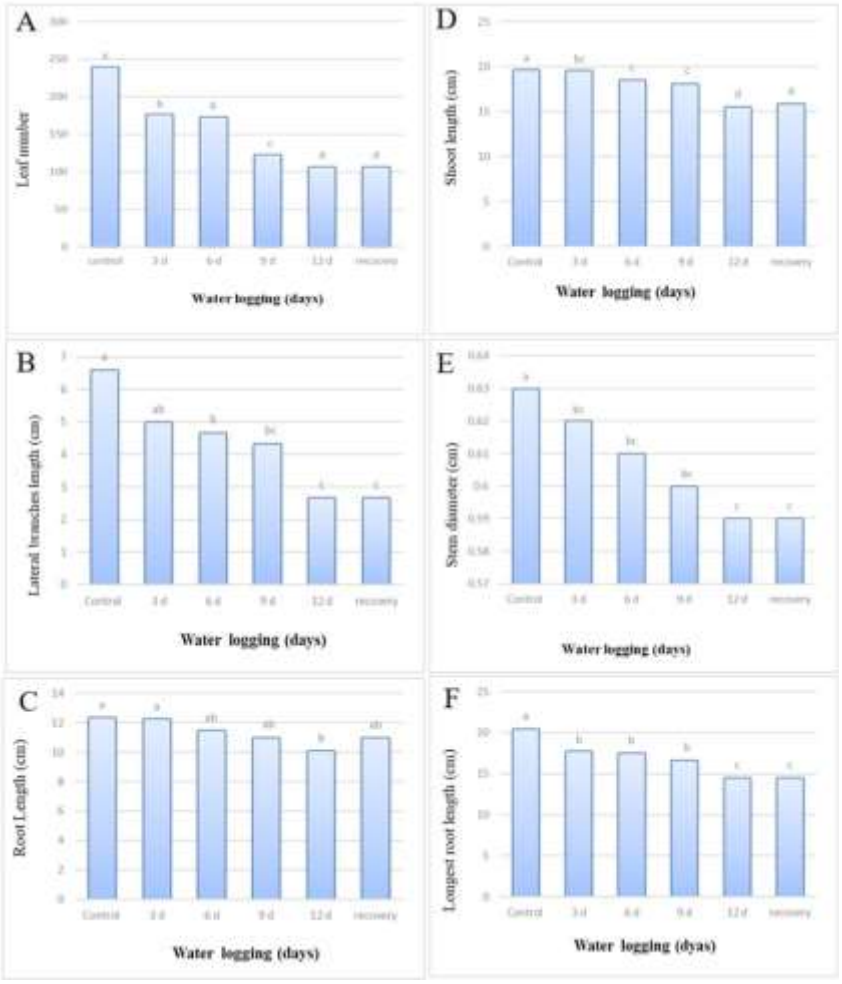

Figure 1- Morphological parameter analysis of *R. chinensis* 'Old Blush' under waterlogging stress and recovery conditions. Letters above the bars indicate significant differences as determined by Duncan's test ($P < 0.05$). Data points in the figure represent the mean values from four biological replicates.

The results indicate that under waterlogging stress, the number of axillary stems decreased with increasing stress duration (Fig. 1). Mean comparison revealed that the greatest shoot length (22.65 cm) occurred in the control (non-flooded) treatment, which did not differ statistically from the recovery treatment (Fig. 1). In contrast, the shortest shoot length (12.86 cm) was observed in the 12-day flooding treatment. Despite the recovery in shoot length (21.59 cm), the Chinese rose plants did not regain their lateral branches. Pardel *et al*. (2014) reported that waterlogging significantly affected total and main

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root length. Similarly, in the present study, total root length decreased with longer waterlogging duration, and root activity in deeper soil layers was severely limited, consistent with the findings of Ganjali *et al*. (2003).

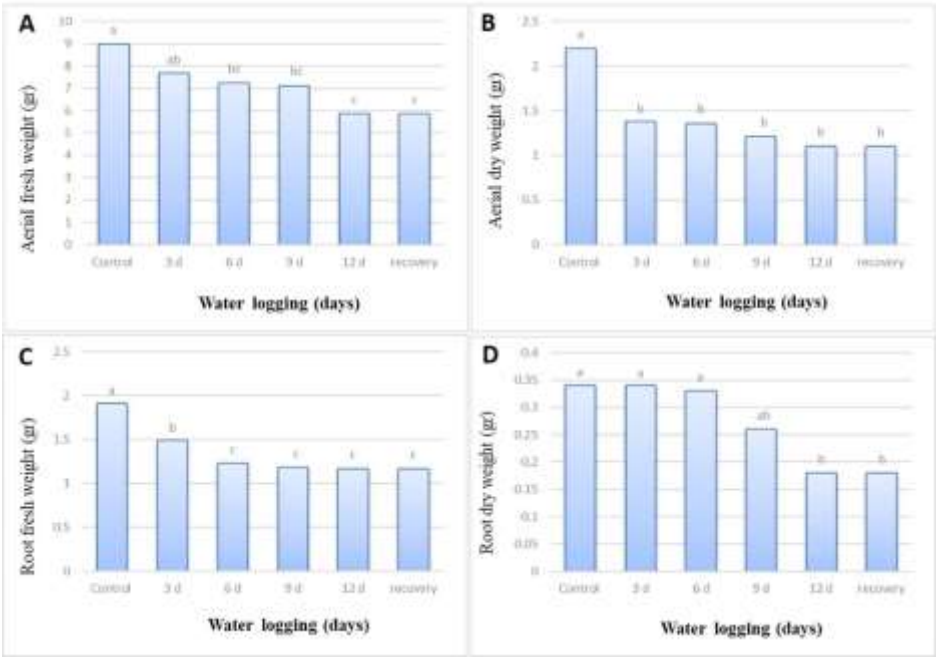

Figure 2. Fresh and dry weight of roots (C, D) and aerial parts (A, B) of *R. chinensis* 'Old Blush' under waterlogging stress and recovery. n = 3. Letters above the bars indicate significant differences as determined by Duncan's test ($P < 0.05$). Data points in the figure represent the mean values from four biological replicates.

Mean comparison revealed that the highest fresh weight of aerial parts (9.005 g) was recorded in the control (non-waterlogged) treatment, with no significant difference from the 3-day waterlogging treatment (Fig. 2). The lowest fresh weight (5.87 g) was observed in the 12-day waterlogging treatment. It was statistically similar to the 6-day, 9-day, and recovery treatments. Studies on *Populus* spp. have shown that waterlogging stress reduces leaf number and size and induces premature leaf abscission (Cao & Conner, 1999; Stewart *et al.,* 2009).

Similarly, the highest shoot dry weight (2.2 g) was found in the control, while the lowest (1.1 g) was observed after 12 days of waterlogging. For roots, the highest fresh weight (1.91 g) was also in the control, and the lowest (1.17 g) was in the 12-day treatment, which was not significantly different from the 6-day and 9-day treatments. Notably, roots in the recovery treatment (1.62 g) did not regain their original biomass (Fig. 2). The results indicated that root length and dry weight remained unchanged relative to the control until day 9 of stress, after which they declined significantly. Interestingly, the root system architecture (RSA) of *R. chinensis* demonstrated considerable plasticity in response to waterlogging. Despite a significant decrease in root fresh and dry weight by day 12, the duration of stress led to an increase in lateral root formation (Fig. 4). While overall root density decreased, the length of some lateral roots at 12 days was notably greater than in other treatments. Furthermore, root growth angles shifted, indicating a tendency to avoid downward growth. These adaptive responses in root growth appear crucial for the survival of *R. chinensis* under waterlogging stress. Similar increases in lateral root formation and RSA changes have been reported in *Arabidopsis* (Harman, 2024). Pardel *et al*. (2014) concluded that the reduction in root traits in *Stevia* was linked to a shift toward less efficient anaerobic respiration. Under such conditions, rapid depletion of root carbohydrate reserves due to low

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energy supply leads to carbohydrate starvation and a severe reduction in root weight, consistent with the findings of the present study.

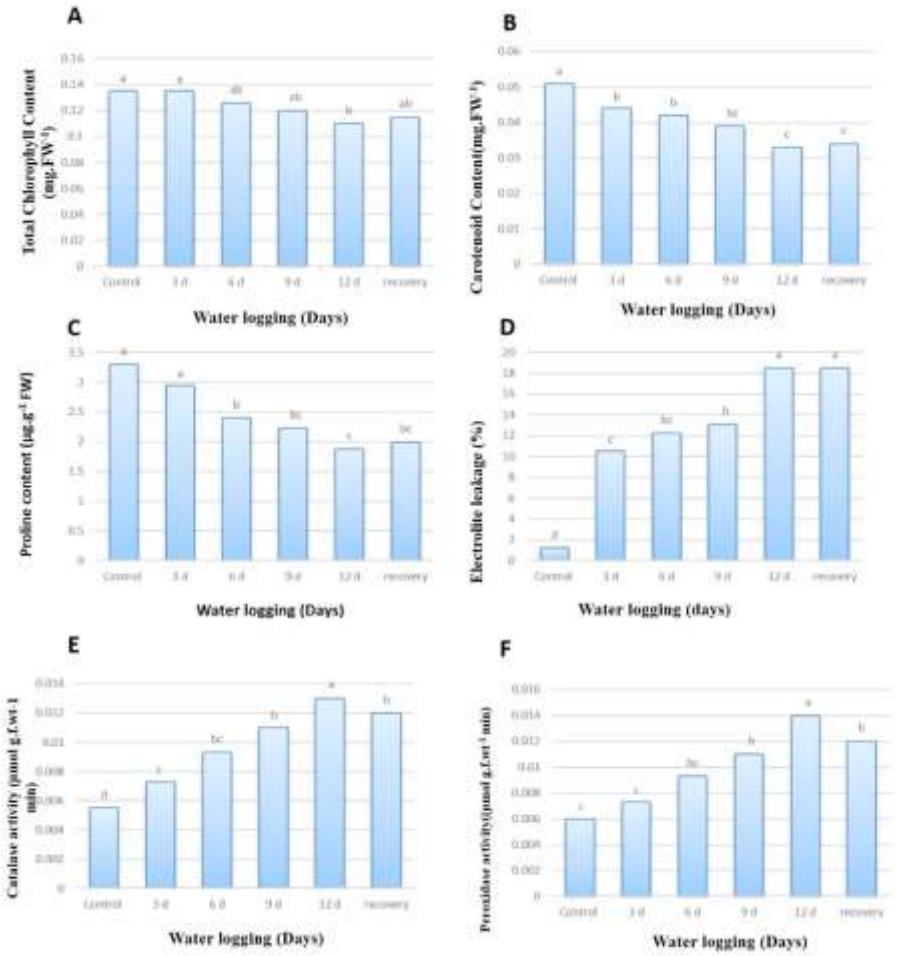

Figure 3. Representative of chlorophyll (A) and carotenoid (B) content, leaf proline content (C), EC (D), catalase and peroxidase (E, F) activity of *R. chinensis* leaf subjected to waterlogging stress. Letters above the bars indicate significant differences as determined by Duncan's test ($P < 0.05$). Data points in the figure represent the mean values from four biological replicates.

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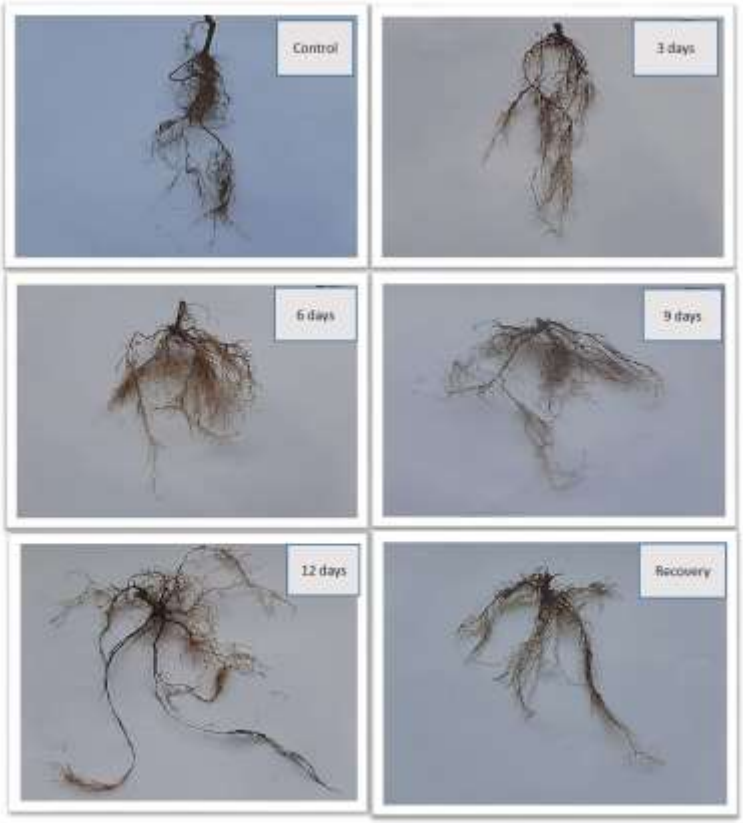

Figure 4. Phenotypic changes in root system architecture of *R. chinensis* 'Old Blush' exposed to waterlogging stress for 3, 6, 9, and 12 days and subsequent recovery. Images were captured with a digital camera.

The highest catalase and peroxidase activities were observed on day 12 of waterlogging, with values of 0.011 and 0.0055 µmol/g, respectively (Fig. 3). Under recovery conditions, the activities of these enzymes partially returned toward control levels. Proline content, a common marker for abiotic stress (Sarmast *et al*., 2015; Rezaei Ghaleh *et al*., 2020), was also quantified. Mean comparison revealed that the highest proline concentration (3.3 µg/g) was found in the control (non-flooded) treatment, which was not statistically different from the 3-day flooding treatment. In contrast, the lowest concentration (1.88 µg/g) was recorded in the 12-day flooding treatment. The observed reduction in proline content under waterlogging stress, contrary to its typical accumulation pattern, can be attributed to a profound energy and metabolic limitation. Proline biosynthesis is an ATP-intensive process. The severe energy deficit induced by root-zone hypoxia, shifting metabolism toward inefficient anaerobic respiration, likely restricts the ATP available for proline synthesis, diverting scarce cellular resources toward core survival pathways. Furthermore, proline synthesis depends on glutamate as a primary precursor. Prolonged stress may lead to a general nitrogen limitation or a disruption in amino acid metabolism, thereby reducing glutamate availability. Consequently, the combined effect of energy scarcity and precursor limitation under these specific experimental conditions provides a plausible physiological explanation for the diminished proline levels, indicating a potential metabolic impairment rather than an absence of stress (Ashraf, 2012).

The research results also indicate that waterlogging severely affects the electrical conductivity of leaf cells and is accompanied by a reduction in chlorophyll content, especially at longer durations (e.g., 12 days). The data demonstrate a direct cause–effect relationship between oxidative stress markers and the antioxidant enzyme response. The progressive rise in electrolyte leakage (EC), confirming membrane damage, was paralleled by a significant increase in the activities of catalase (CAT) and peroxidase

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(POX). Specifically, the peak in EC at 12 days of stress coincided with the maximum activity of these enzymes. This correlation indicates that the upregulation of CAT and POX was a direct biochemical countermeasure to scavenge hydrogen peroxide and other reactive oxygen species, whose accumulation directly causes the lipid peroxidation reflected in the EC measurements. Therefore, these changes in enzyme activity are not merely associated with stress but represent a functional response to the quantified oxidative damage.

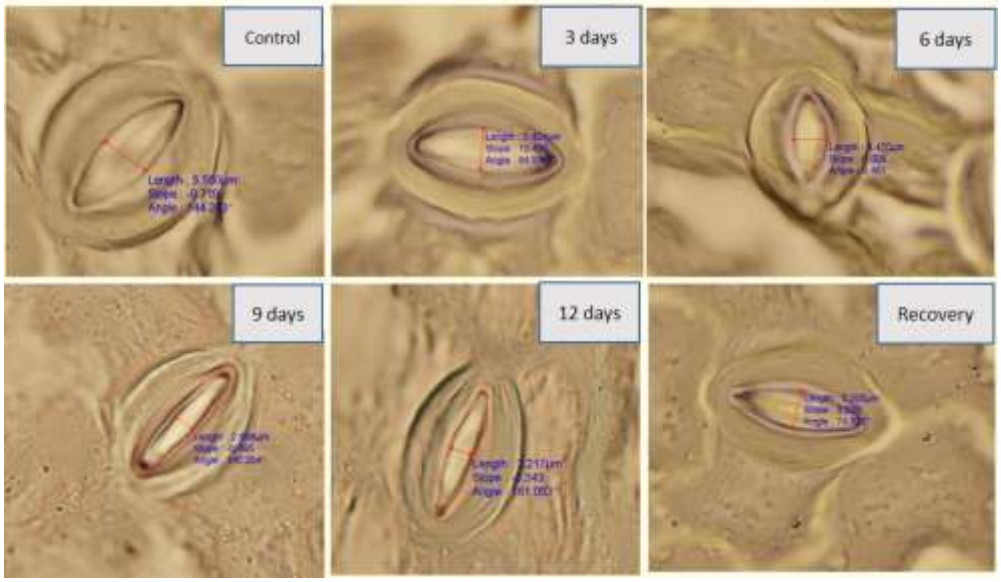

Figure 5. **Measurement of stomatal aperture (width) in *Rosa chinensis* 'Old Blush' under waterlogging stress and subsequent recovery. Photomicrographs were taken using an optical microscope at 40× magnification.**

To understand the reason for chlorophyll reduction, stomatal conductance was also monitored (Fig. 5). The results showed that as the duration of waterlogging stress increased, stomatal conductance shifted from a fully open state in the control plants to a nearly closed state under the most prolonged stress. In recovery treatments, stomatal apertures reopened. These findings align with previous studies on waterlogging stress. For example, Linkmer *et al*. (1998) found that stomatal conductance and the rate of leaf photosynthesis decreased in soybean. Similarly, Ahmed *et al*. (2002) reported reduced stomatal conductance and water use efficiency in waterlogged peanut plants. Ahmad *et al*. (2002) further noted that flooding-induced oxidative stress causes severe photosynthetic damage in mung bean plants due to reactive oxygen species and toxic substances. Plants combat this through highly efficient antioxidant defense systems, which protect against free radicals (Tiryakioğlu *et al*., 2015). The increased activities of catalase and peroxidase observed in this study (Fig. 3) are consistent with this protective mechanism. A key methodological consideration is that soil oxygen dynamics were not directly quantified in this pot-based system. Although crown submersion is a standard and effective method for inducing root hypoxia, the lack of direct oxygen or redox potential measurements means our discussion of hypoxia-triggered pathways (e.g., ethylene accumulation, anaerobic metabolism) is inferred from plant responses rather than being correlated with direct edaphic measurements. Future studies incorporating such soil physico-chemical data would strengthen the mechanistic understanding of the observed responses.

Conclusion

In summary, due to the lack of energy available in the waterlogged conditions in the plant, the root tissues quickly consumed their stored carbohydrates, leading to a sharp decrease in the fresh and dry weights of the roots. On the other hand, these conditions led to a decrease in stomatal conductance and

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a lack of carbon dioxide for nutrient synthesis, which was accompanied by membrane damage, increased antioxidant enzyme activity, and changes in root system plasticity to reduce the accumulation of ROS.

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