



MAXWELL PEREIRA DE PÁDUA

**GRADATIVE EFFECT OF PHOSPHORUS IN STOMATAL
TRAITS REGULATED BY ETHYLENE AND ABA
AFFECTING PHOTOSYNTHESIS AND GROWTH OF
Solanum lycopersicum L.**

**LAVRAS – MG
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Tese apresentada à Universidade Federal de
Lavras, como parte das exigências do
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Aplicada, área de concentração em Botânica
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**EFEITO GRADATIVO DO FÓSFORO EM CARACTERÍSTICAS
ESTOMÁTICAS REGULADAS POR ETILENO E ABA AFETAM A
FOTOSSÍNTESE E O CRESCIMENTO DE *Solanum lycopersicum* L.**

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RESUMO GERAL

Este estudo investiga os efeitos do excesso de fósforo (P) no fechamento estomático e no crescimento de *Solanum lycopersicum* L., com foco nos efeitos estomáticos sinalizados pelos fitormônios etileno e ácido abscísico (ABA). O uso excessivo de fertilizantes fosfatados em práticas agrícolas modernas pode aumentar as concentrações de P no solo, levando a impactos ambientais negativos, como a eutrofização de solos e águas, prejudicando o ambiente, seus organismos e mesmo atividades humanas. Apesar de vários trabalhos investigarem os efeitos da deficiência de P para as plantas, os efeitos do excesso de P para as plantas ainda são pouco compreendidos. Pesquisas têm mostrado que plantas expostas a concentrações excessivas de P apresentam reduções na condutância estomática, fotossíntese e crescimento. O primeiro artigo desta tese examina o fechamento estomático desencadeado por fosfato e mediado por etileno em mutantes de *S. lycopersicum*. Foi observado que a aplicação exógena de P diretamente na epiderme foliar promoveu o fechamento estomático e reduziu a condutância estomática em todos os genótipos estudados, com o mutante *Notabilis* (deficiente em ABA) sendo o mais responsivo e o mutante *Never ripe* (baixa sensibilidade ao etileno) sendo o menos responsivo. O segundo artigo investiga como o excesso de P reduz o crescimento em *S. lycopersicum*, destacando o papel do fechamento estomático mediado por etileno e ABA. O excesso de P causou redução na condutância estomática, transpiração e crescimento das plantas, mas não causou mudanças significativas nos parâmetros fotoquímicos em *S. lycopersicum*, indicando que os efeitos negativos do excesso de P podem estar relacionados a limitações na aquisição de CO₂. Além disso, o excesso de P aumentou o conteúdo de H₂O₂ nas folhas. As respostas variaram entre os genótipos, sugerindo que a sinalização de etileno e ABA é crucial para as respostas das plantas ao excesso de P. Os resultados deste estudo são fundamentais para compreender os impactos do excesso de fósforo nas plantas e podem contribuir para práticas agrícolas mais sustentáveis, minimizando os riscos ambientais e maximizando a eficiência do uso de fertilizantes.

Palavras-chave: ácido abscísico; etileno; célula-guarda; fósforo; fechamento estomático; tomate mutante.

GENERAL ABSTRACT

This study investigates the effects of excess phosphorus (P) on the stomatal closure and growth of *Solanum lycopersicum* L., focusing on the stomatal effects signaled by the phytohormones ethylene and abscisic acid (ABA). The excessive use of phosphate fertilizers in modern agricultural practices can increase P concentrations in the soil, leading to negative environmental impacts, such as the eutrophication of soils and waters, damaging the environment, its organisms and even human activities. Although several studies have investigated the effects of P deficiency on plants, the effects of excess P on plants are still poorly understood. Research has shown that plants exposed to excessive concentrations of P show reductions in stomatal conductance, photosynthesis and growth. The first article in this thesis examines phosphate-triggered and ethylene-mediated stomatal closure in *S. lycopersicum* mutants. It was observed that exogenous application of P directly to the leaf epidermis promoted stomatal closure and reduced stomatal conductance in all the genotypes studied, with the *Notabilis* mutant (deficient in ABA) being the most responsive and the *Never ripe* mutant (low sensitivity to ethylene) being the least responsive. The second article investigates how excess P reduces growth in *S. lycopersicum*, highlighting the role of stomatal closure mediated by ethylene and ABA. Excess P caused a reduction in stomatal conductance, transpiration and plant growth, but did not cause significant changes in photochemical parameters in *S. lycopersicum*, indicating that the negative effects of excess P may be related to limitations in CO₂ acquisition. In addition, excess P increased the H₂O₂ content in the leaves. The responses varied between genotypes, suggesting that ethylene and ABA signalling are crucial to plant responses to excess P. The results of this study are fundamental to understanding the impacts of excess phosphorus on plants and can contribute to more sustainable agricultural practices, minimizing environmental risks and maximizing the efficiency of fertilizer use.

Keywords: abscisic acid; ethylene; guard cells; phosphorus; stomatal closure; tomato mutants.

INDICADORES DE IMPACTOS

A relação do excesso de fósforo (P) com os fitormônios etileno e ácido abscísico (ABA) e a influência destes compostos no fechamento estomático, bem como nas características de crescimento e fotossíntese de plantas, é um tema fundamental para a anatomia e fisiologia vegetal. A adubação fosfatada é de extrema importância para a produção agrícola, mas pode gerar impactos ambientais e custos de produção. Essa pesquisa destaca o papel do etileno e do ABA no fechamento estomático induzido pelo P, mostrando que o excesso de P promove o fechamento dos estômatos, reduzindo a condutância estomática e, consequentemente, diminuindo a fotossíntese e o crescimento das plantas. Compreender estes efeitos do P pode levar ao desenvolvimento de novas tecnologias e práticas agrícolas mais eficientes, reduzindo o uso excessivo de fertilizantes e promovendo uma agricultura sustentável. A pesquisa também pode inspirar o melhoramento genético de plantas com o objetivo de otimizar a eficiência na absorção de P e reduzir os efeitos negativos do seu excesso. Este estudo se alinha a alguns dos Objetivos do Desenvolvimento Sustentável (ODS) da ONU. Socialmente, a compreensão dos efeitos do P é crucial para promover práticas agrícolas sustentáveis, que podem melhorar a saúde pública ao evitar a eutrofização de corpos d'água e garantir a segurança alimentar, alinhando-se ao ODS 2 - Fome Zero e Agricultura Sustentável. Tecnicamente, os estudos avançam o conhecimento sobre a fisiologia das plantas, destacando a importância dos fitormônios etileno e ABA na regulação do fechamento estomático em plantas submetidas ao excesso de P. Este conhecimento pode levar ao desenvolvimento de cultivares mais eficientes, atendendo ao ODS 9 - Indústria, Inovação e Infraestrutura. Economicamente, a pesquisa demonstra que o uso inadequado de fertilizantes fosfatados pode reduzir a produtividade das culturas, aumentar os custos de produção e impactar negativamente a renda dos agricultores. Por inspirar a promoção de práticas agrícolas sustentáveis que podem reduzir custos e melhorar a produtividade, a pesquisa contribui para o ODS 12 - Consumo e Produção Responsáveis.

IMPACT INDICATORS

The relationship between excess phosphorus (P) and the phytohormones ethylene and abscisic acid (ABA) and the influence of these compounds on stomatal closure, as well as on plant growth and photosynthesis characteristics, is a fundamental topic for plant anatomy and physiology. Phosphate fertilization is extremely important for agricultural production, but it can generate environmental impacts and production costs. The research highlights the role of ethylene and ABA in P-induced stomatal closure, showing that excess P promotes stomatal closure, reducing stomatal conductance and, consequently, decreasing photosynthesis and plant growth. Understanding these effects of P can lead to the development of new technologies and more efficient agricultural practices, reducing the excessive use of fertilizers and promoting sustainable agriculture. The research could also inspire the genetic improvement of plants with the aim of optimizing their efficiency in absorbing P and reducing the negative effects of its excess. This study aligns with some of the United Nations Sustainable Development Goals (SDGs). Socially, understanding the effects of P is crucial to promoting sustainable agricultural practices, which can improve public health by preventing the eutrophication of water bodies and guaranteeing food security, in line with SDG 2 - Zero Hunger and Sustainable Agriculture. Technically, the studies advance knowledge about plant physiology, highlighting the importance of the phytohormones ethylene and ABA in regulating stomatal closure in plants subjected to excess P. This knowledge could lead to the development of more efficient cultivars, in line with SDG 9 - Industry, Innovation and Infrastructure. Economically, the research shows that the inappropriate use of phosphate fertilizers can reduce crop yields, increase production costs and negatively impact farmers incomes. By inspiring the promotion of sustainable agricultural practices that can reduce costs and improve productivity, the research contributes to SDG 12 - Responsible Consumption and Production.

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PRIMEIRA PARTE

1 INTRODUÇÃO GERAL

O uso excessivo de fertilizantes fosfatados na agricultura e o despejo de resíduos orgânicos podem levar a um aumento nas concentrações de fósforo (P) nos solos. Este acúmulo pode resultar em vários impactos negativos, incluindo a lixiviação de P, causando eutrofização de solos e ambientes aquáticos (Sharpley e Tunney, 2000; Smith e Schindler, 2009). Ambientes podem ser considerados eutróficos em relação ao P quando possuem concentrações de 0,2 mM (White *et al.*, 2000). Concentrações de P de 0,6 mM e acima, podem ser consideradas hipertróficas (Steinbachová-Vojtísková *et al.*, 2006; Santos *et al.*, 2015) e ambientes que são eutrofizados pelo excesso de P são frequentemente associados ao crescimento excessivo de plantas aquáticas e espécies invasoras que podem causar problemas ambientais como a redução da biodiversidade e dificuldade de uso destas áreas (Esteves, 2011; Zamparas e Zacharias, 2014; Santos *et al.*, 2015).

Por outro lado, o P é um elemento essencial para o crescimento e desenvolvimento das plantas, desempenhando um papel importante no metabolismo energético, fotossíntese e síntese de diversas biomoléculas (Raghothama e Karthikeyan, 2005; Taiz *et al.*, 2017). Em razão da importância do P para o metabolismo básico e para a composição estrutural este é considerado um nutriente essencial e é altamente demandado pelas plantas podendo ser classificado como um macronutriente e configura, aproximadamente 0,2% da massa seca das plantas (Taiz *et al.*, 2017; Salisbury, 1992). Portanto, em ambientes com baixa disponibilidade de P, a adubação fosfatada é uma importante técnica que pode favorecer a produção agrícola. Entretanto, é preciso levar em consideração os impactos ambientais de uma possível aplicação excessiva de fosfato, além disso, o excesso de P pode causar efeitos negativos em plantas sensíveis, por isso, é preciso caracterizar e entender os efeitos do excesso de P nas plantas. Neste contexto, compreender esses efeitos é fundamental para o desenvolvimento de práticas agrícolas sustentáveis que minimizem os riscos ambientais e maximizem a eficiência do uso de fertilizantes.

Apesar deste contexto descrito e de vários trabalhos investigarem os problemas da deficiência de P para as plantas, o efeito do excesso deste elemento nas plantas foi pouco explorado. Concentrações adequadas de P são essenciais para o crescimento e desenvolvimento das plantas, e seu excesso pode prejudicar a absorção de outros elementos como o Zn, causando desequilíbrio nutricional nas plantas (Zhang *et al.*, 2012). Trabalhos demonstram que o excesso de P pode promover redução de crescimento, taxa fotossintética e produtividade (Campos-

Soriano *et al.*, 2020; Santos *et al.*, 2015; Zakrzewska *et al.*, 2020). Além disso, recentemente reduções nas trocas gasosas, parênquima clorofiliano e na atividade meristemática foram descritos na literatura em razão de concentrações hipertróficas de P (Santos *et al.*, 2015; Corrêa *et al.*, 2017; Santos *et al.*, 2021). As razões para tais efeitos ainda são incertas por conta da limitada literatura a respeito. Contudo, recentemente um trabalho demonstrou a relação do excesso de P com a produção do fitormônio etileno (Shukla *et al.*, 2017). Este fitormônio é reconhecidamente um sinalizador para estresses ambientais e pode levar a síntese de outros compostos como o ácido abscísico (ABA) que, por sua vez também pode promover respostas fatores ambientais em situações adversas (Grossmann e Hansen, 2001). Entretanto, a investigação sobre a possibilidade das limitações fotossintéticas e de crescimento nas plantas expostas ao excesso de fósforo até o momento não foi associada à produção de fitormônios. O tema central deste estudo, portanto é esta conexão entre o excesso de P e a possível sinalização pelo etileno e/ou ABA. A partir deste tema, diferentes hipóteses foram levantadas e experimentos foram realizados, utilizando tomateiro como planta modelo para auxiliar no entendimento destas questões.

O tomate, *Solanum lycopersicum* L. (Solanaceae) é uma das mais importantes culturas mundiais, sendo que a produção total em 2023 foi de 192,3 milhões de toneladas e o Brasil é o nono maior produtor mundial com 3,64 milhões de toneladas em 2023 (FAO, 2025). Além de sua importância agroeconômica, o tomateiro é um importante modelo para estudar processos biológicos. Neste trabalho, para investigar possíveis respostas do excesso de P, foi utilizado *Solanum lycopersicum* L. cv. Micro-Tom (MT), pois é um modelo biológico ideal devido ao seu ciclo de vida rápido e tamanho reduzido, facilitando experimentos em condições controladas. Estas plantas podem crescer em laboratório com mesma estrutura mínima demandada para *Arabidopsis* (Lima *et al.*, 2004; Carvalho *et al.*, 2011).

Foram utilizados genótipos específicos de *S. lycopersicum* que apresentam modificações metabólicas que os tornam eficientes modelos para este trabalho. Estes genótipos possuem tamanho reduzido em comparação com aqueles destinados ao cultivo comercial e apresentam outras características interessantes como modelo para experimentos (Carvalho *et al.*, 2011). Dentre estes, foi utilizado o genótipo *Never ripe* (Nr), que demonstra baixa sensibilidade ao etileno devido a uma mutação que expressa uma deficiência no receptor LeETR3 que impossibilita a ligação do etileno (Wilkinson *et al.*, 1995). Devido a essa mutação, essas plantas não apresentam respostas típicas ao etileno, apresentam atraso na senescência e abscisão das pétalas e os frutos não amadurecem (Hackett *et al.*, 2000; Carvalho *et al.*, 2011)

Outro genótipo utilizado neste trabalho foi o *Notabilis (not)* que apresenta deficiência na produção e acúmulo de ácido abscísico (ABA) por ser defectivo no gene 9-cis-epoxycarotenoid dioxygenase (NCED), sendo uma enzima de clivagem de carotenóides, necessários na síntese de ABA, além desses, também foi utilizado o genótipo *Wild Type (WT)* que não apresenta mutações hormonais (Burbidge *et al.*, 1999; Carvalho *et al.*, 2011).

A relação do excesso de P na produção de fitormônios etileno e ABA e a influência destes compostos no fechamento estomático bem como características de crescimento e fotossíntese de plantas são temas fundamentais para a agronomia e fisiologia vegetal. Sendo a adubação fosfatada de extrema importância para a produção agrícola, esta pode gerar impactos ambientais além de gerar altos custos de produção. Entender o efeito do excesso de fosfato nas plantas poderá gerar sistemas mais eficientes de produção com menores custos e impactos ambientais. Portanto, este trabalho visa contribuir para o melhor entendimento do efeito do excesso de P para as plantas.

Nesse sentido, o primeiro objetivo deste estudo foi avaliar o efeito do excesso de P no fechamento estomático de três genótipos de *S. lycopersicum* L.; *WT*, *not* (deficiente em ABA) e *Nr* (baixa sensibilidade ao etileno), e investigar as consequências deste efeito para a estrutura e fisiologia estomática. O segundo objetivo foi investigar o efeito de diferentes concentrações de P nas respostas estomáticas, parâmetros fotoquímicos, crescimento e desenvolvimento desses mesmos genótipos de *S. lycopersicum*.

REFERÊNCIAS

- CARVALHO, R. F.; CAMPOS, M. L.; PINO, L. E.; CRESTANA, S. L.; ZSÖGÖN, A.; LIMA, J. E.; BENEDITO, V. A.; PERES, L. E. P. Convergence of developmental mutants into a single tomato model system: 'Micro-Tom' as an effective toolkit for plant development research. **Plant Methods**, v. 7, 18, 2011. <https://doi.org/10.1186/1746-4811-7-18>
- BURBIDGE, A.; GRIEVE, T. M.; JACKSON, A.; THOMPSON, A.; MCCARTY, D. R.; TAYLOR, I. B. Characterization of the ABA deficient tomato mutant notabilis and its relationship with maize Vp14. **Plant Journal**, v. 17, p. 427-431, 1999. <https://doi.org/10.1046/j.1365-3113x.1999.00386.x>
- CAMPOS-SORIANO, L.; BUNDÓ, M.; BACH-PAGES, M.; SHIANG, S-F.; CHIOU, T-J.; SEGUNDO, B. S. Phosphate excess increases susceptibility to pathogen infection in rice. **Molecular Plant Pathology**, v. 21, p. 555-570, 2020. <https://doi.org/10.1111/mpp.12916>
- CORRÊA, F. F.; PEREIRA, M. P.; KLOSS, R. B.; CASTRO, E. M.; PEREIRA, F. J. Leaf ontogeny and meristem activity of *Typha domingensis* Pers. (Typhaceae) under different phosphate concentrations. **Aquatic Botany**, v. 136, p. 43-51, 2017. <https://doi.org/10.1016/j.aquabot.2016.09.007>
- ESTEVEES, F. A. **Fundamentos de Limnologia**. Rio de Janeiro: Editora Interciência-FINEP, 3ª. ed, 2011, 568 p.
- FAO (Food and Agriculture Organization of the United Nations). FAOSTAT: Crops and livestock products: tomatoes. Disponível em: <https://www.fao.org/faostat/en/#data/QCL/visualize> acesso em 15-01-2025.
- GROSSMANN K, HANSEN H. Ethylene-triggered abscisic acid: a principle in plant growth regulation? **Physiol Plant**, v. 113, p. 9-14, 2001. <http://dx.doi.org/10.1034/j.1399-3054.2001.1130102.x>
- LIMA, J.E., CARVALHO, R.F., TULMANN NETO, A., FIGUEIRA, A., PERES, L.E.P. Micro-MsK: a tomato genotype with miniature size, short life cycle, and improved in vitro shoot regeneration. **Plant Science**, v. 167, p. 753-757, 2004.
- HACKETT, R. M., HO, C. W., LIN, Z. F., FOOTE, H. C. C., FRAY, R. G.; GRIERSON, D. Antisense inhibition of the Nr gene restores normal ripening to the tomato Never-ripe mutant, consistent with the ethylene receptor-inhibition model. **Plant Physiology**, 124, p. 1079-1086, 2000. <https://doi.org/10.1104%2Fpp.124.3.1079>
- RAGHOTHAMA, K. G.; KARTHIKEYAN, A. S. Phosphate acquisition. **Plant and Soil**, v. 274, p. 37-49, 2005. <https://doi.org/10.1007/s11104-004-2005-6>
- SALISBURY, Frank B. **Plant physiology**. 4.ed Belmont, California: Wadsworth, 1992. 682 p.

SANTOS, J. C. B.; CASTRO, E. M.; DUARTE, V. P.; PEREIRA, M. P.; CORRÊA, F. F.; PEREIRA, F. J. Root growth and anatomy of *Typha domingensis* Pers. related to phosphorus availability. **Acta Limnologica Brasiliensia**, v. 33, e19, 2021.

<https://doi.org/10.1590/S2179-975X2120>

SANTOS, K. R.; PEREIRA, M. P.; FERREIRA, A. C. G.; RODRIGUES, L. C. A.; CASTRO, E. M.; CORRÊA, F. F.; PEREIRA, F. J. *Typha domingensis* Pers. Growth responses to leaf anatomy and photosynthesis as influenced by phosphorus. **Aquatic Botany**, v. 122, p. 47-53, 2015. <https://doi.org/10.1016/j.aquabot.2015.01.007>

SHUKLA, D.; RINEHART, C. A.; SAHI, S. V. Comprehensive study of excess phosphate response reveals ethylene mediated signaling that negatively regulates plant growth and development. **Scientific Reports**, v. 7, 3074, 2017.

<https://doi.org/10.1038/s41598-017-03061-9>

SHARPLEY, N. A.; TUNNEY, H. Phosphorus research strategies to meet agricultural and environmental challenges of the 21st century. **J Environ Qual**, v. 29, p. 176-181, 2000.

<https://doi.org/10.2134/jeq2000.00472425002900010022x>

SMITH, V. H.; SCHINDLER, D. W. Eutrophication science: where do we go from here? **Trends in Ecology and Evolution**, v. 24, p. 201-207, 2009.

<https://doi.org/10.1016/j.tree.2008.11.009>

STEINBACHOVÁ-VOJTÍŠKOVÁ, L.; TYLOVÁ, E.; SOUKUP, A.; NONICKÁ, H.; VOTRUBOVÁ, O.; LIPAVSKÁ, H.; ČÍŽKOVÁ, H. Influence of nutrient supply on growth carbohydrate, and nitrogen metabolic relations in *Typha angustifolia*.

Environmental and Experimental Botany, v. 57, p. 246-257, 2006.

<https://doi.org/10.1016/j.envexpbot.2005.06.003>

TAIZ, L.; ZEIGER, E.; MOLLER, I. M.; MURPHY, A. **Fisiologia e desenvolvimento vegetal**. 6. ed. Porto Alegre: ArtMed, 2017. E-book. (1 recurso online). ISBN 9788582713679.

WHITE, J. S.; BAYLEY, S. R.; CURTIS, P. J. Sediment storage of phosphorus in a northern prairie wetland receiving municipal and agro-industrial wastewater. **Ecological Engineering**, v. 14, p. 127-138, 2000. [https://doi.org/10.1016/S0925-8574\(99\)00024-5](https://doi.org/10.1016/S0925-8574(99)00024-5)

WILKINSON, J. Q.; LANAHAN, M. B.; YEN, H.; GIOVANNONI, J. J.; KLEE, H. J. An ethylene-inducible component of signal transduction encoded by never-ripe. **Science**, 270/5243, 1807-1809, 1995. <https://doi.org/10.1126/science.270.5243.1807>

ZAKRZEWSKA, J.; MITROVIĆ, A. L.; MUTAVDŽIĆ, D.; DUČIĆ, T.; RADOTIĆ, K. Phosphorus homeostasis in *Populus alba* L. under excess phosphate conditions, assessed by ³¹P nuclear magnetic resonance spectroscopy and X-ray microfluorescence. **Environmental Science and Pollution Research**, v. 27, p. 3320–3328, 2020.

<https://doi.org/10.1007/s11356-019-07200-9>

ZAMPARAS, M.; ZACHARIAS, I. Restoration of eutrophic freshwater by managing

internal nutrient loads. **Science of Total Environment**, v. 496, p. 551-562, 2014.
<https://doi.org/10.1016/j.scitotenv.2014.07.076>

ZHANG, Y-Q.; DENG, Y.; CHEN, R-Y.; CUI, Z-L.; CHEN, X-P.; YOST, R.; ZHANG, F-S.; ZOU, C-Q. The reduction in zinc concentration of wheat grain upon increased phosphorus-fertilization and its mitigation by foliar zinc application. **Plant Soil**, v. 361, 143-152, 2012.
<https://doi.org/10.1007/s11104-012-1238-z>

SEGUNDA PARTE - ARTIGOS

**ARTIGO 1 – Phosphate-triggered and ethylene-mediated stomatal closing on the leaf epidermis
of *Solanum lycopersicum* L. mutants**

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Phosphate-triggered and ethylene-mediated stomatal closing on the leaf epidermis of *Solanum lycopersicum* L. mutants

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Abstract

This study aimed to evaluate the effect of exogenous P application on the stomatal physiology of three tomato genotypes: the wild-type (*Wt*), the ABA-deficient mutant *notabilis* (*not*), and the low ethylene sensitivity mutant *Never ripe* (*Nr*). The tomato genotypes were grown in a growth room for 60 days using Hoagland's nutrient solution, leaves were then collected, and fragments of the leaf epidermis were sampled and exposed to exogenous solutions of ABA and P for 10 min. The percentage of open and closed stomata, stomatal density, length and width, and pore area were evaluated and the maximum stomatal conductance was estimated using stomata data. ABA promoted stomatal closure in all the genotypes, and the *notabilis* genotype showed a lower response. Stomatal density was not expected to be influenced by ABA or P from a direct epidermal application on already developed leaves while it was higher in *notabilis* than in *Nr* and *Wt* genotypes. P promoted a higher percentage of closed stomata in all genotypes, with *notabilis* being the most responsive and *Nr* the lowest. P reduced the pore area and proportion in all genotypes, with *notabilis* being the most responsive and *Nr* the least. P increased the stomatal length and a reduction in stomatal width in all genotypes, with *notabilis* being the most responsive. P reduced the stomatal conductance in all genotypes with *notabilis* being the most responsive and *Nr* the less responsive. Therefore, excessive P application on the leaf epidermis of *S. lycopersicum* promotes stomatal closure and reduction of stomatal conductance, and the results indicate that this closure is directly related to ethylene signaling in the leaf epidermis.

Keywords: Tomato mutants, Phosphate, Stomatal physiology, Abscissic acid, Stomatal conductance.

Introduction

P is an essential macronutrient for plants and has many important functions, including as a structural component of DNA, RNA, ATP, ADP, and membrane phospholipids (Raghothama and Karthikeyan 2005), participating from the coding of genes and their expression to energy metabolism, just to name a handful. Due to its importance, this element is widely studied in terms of its deficiency in plants, which can lead to a reduction in growth (Gruber et al. 2013) and an investment in the root system to favor the acquisition of the element via absorption from roots (Raghothama and Karthikeyan 2005; Liu 2021). Moreover, P limitation promotes an increase in the vascular tissues of the roots (Santos et al. 2021). The limitation of P may result in limitations in gas exchange and photosynthesis (Santos et al. 2015; Kayoumu et al. 2023), negatively affecting growth.

The responses of P deficiency are well known in plants, but an aspect that remains insufficiently studied is the effect of P excess. Despite the paucity of studies on P excess, recent research suggests that P excess, particularly in a eutrophic/hypertrophic situation, above 0.6 mM can cause a reduction in stomatal conductance, transpiration and photosynthesis (Santos et al. 2015); a reduction in the development of the root system, with shorter roots and lower mass (Santos et al. 2021); and a reduction in the activity of leaf meristems, particularly in the ground meristem that gives rise to the chlorophyll parenchyma (Corrêa et al. 2017). These three cited studies focused on *Typha domingensis* Pers., which is considered a P-tolerant species. Excess P can also decrease the resistance to infections in *Oryza sativa* L., increasing infection by ascomycete fungi (Campos-Soriano et al. 2020). When in excess, P can also reduce the growth and development of *Populus alba* L. (Zakrzewska et al. 2020). It is also estimated that in *Solanum lycocarpum* L., P concentrations above 1% in dry plant biomass can cause toxicity (Jones 1998). Therefore, above a certain limit, this macronutrient causes toxicity in plants. This subject needs to be investigated because many environments suffer P eutrophication due to sewage dumping and other human activities.

It is known that P can impair the acquisition of some nutrients like Zn (Nadeem et al. 2024; Shukla et al. 2017), Mo (Wurzburger et al. 2012), Ca, Fe, and Mn (Shukla et al. 2017). However, the evidence of the negative effect of P excess that is reported in the literature is insufficient for understanding the negative effect of this element on the stomatal conductance for instance. What most caught our attention was the reduction in leaf gas exchange in *T. domingensis* (Santos et al. 2015) since P is essential for the structure of many molecules involved in photosynthesis, such as NADPH, ATP, and several phosphate carbohydrates of the Calvin–Benson cycle and competition with Zn and Mo could not explain the reduced photosynthesis because these are micronutrients. Because gas exchange analyses with infrared gas exchange analyzers measuring the capture of CO₂ in the leaf showed a reduction in stomatal conductance (Santos et al. 2015); we hypothesized that the limitation in photosynthesis in these plants is related to the effect of P on stomatal closure, which would limit CO₂ uptake and, consequently, photosynthesis and growth.

Regarding this hypothesis, there was not much information on how P might affect stomatal closure until the publication of the study by Shukla et al. (2017), who demonstrated that excess P promotes ethylene production in *Arabidopsis thaliana* L. with negative effects on plant growth. Ethylene production in *A. thaliana* may be related to nutrient imbalance since

decreased Ca, Fe, Mn, and Zn contents were observed under excessive P although details of the mechanism for ethylene production are still unclear (Shukla et al. (2017)). This study allowed us to better develop our hypothesis and postulate that P excess leads to ethylene synthesis and that this phytohormone in turn promotes stomatal closure, limiting stomatal conductance and photosynthesis. This hypothesis is further supported by the fact that ethylene is a phytohormone that can promote stomatal closure, binding to membrane receptors on guard cells and producing H₂O₂, which signals closure (Desikan et al. 2006). We therefore chose to work with mutant genotypes of *S. lycopersicum* because the species can demonstrate sensitivity to excess P. We used wild type (*Wt*) genotypes, a mutant genotype that has low sensitivity to ethylene never ripe (*Nr*) and another that is deficient in ABA production *notabilis* (*not*) (Burbidge et al. 1999; Hackett et al. 2000; Carvalho et al. 2011).

In summary, the objective of this study was to evaluate the effect of exogenous P application to leaf epidermis on stomatal closure of three of *Solanum lycopersicum* L. genotypes: *Wt*, ABA-deficient (*notabilis*) and low ethylene sensitivity (*Nr*) and to investigate the consequences of this effect for stomatal structure and physiology.

Materials and Methods

Plant material

In this study, three genotypes of *Solanum lycopersicum* L. cv. Micro-Tom (MT) were used: the *wild-type* genotype (*Wt*) as a control; the mutant *Never ripe* (*Nr*), which is defective in the ethylene receptor *LeETR3* and expresses low sensitivity to ethylene; and the mutant *notabilis* (*not*), which is defective in the carotenoid-cleaving enzyme 9-cis-epoxycarotenoid dioxygenase and therefore defective in the production and accumulation of abscisic acid (ABA) (Carvalho et al. 2011).

The seeds were sown directly into 400 ml pots containing 350 ml of washed medium sand and were supplemented weekly with Hoagland and Arnon (1950) nutritive solution and irrigated every two days until the maximum water holding capacity was reached. The study was conducted at the Laboratory of Biotechnology and Genotoxicity (BIOGEN) of the Federal University of Alfenas – UNIFAL – Alfenas, Minas Gerais, Brazil. A growth room with a temperature of 22 ± 2 °C, a photoperiod of 12 hours and an irradiance of 40 W m⁻² were used.

Experimental design

Experiment 1 - Effect of exogenous ABA and genotypes

The first experiment was conducted with a completely randomized design in a 3 x 2 factorial design, with three *S. lycopersicum* genotypes (*Wt*, *Nr*, and *notabilis*) and two solutions: distilled water and 10 mM abscisic acid (ABA) in phosphate buffer 0.1 M pH 6.5. Experiments were performed with nine replicates ($n = 54$) of one plant. Two drops of each solution were placed over the slide and then leaf epidermal sections were transferred to these drops, covered with coverslips, and let to react at room temperature (22 ± 2 °C) for 10 min being immediately photographed. Two leaves per plant were analyzed, with one slide per leaf and three fields per slide, totaling 324 sample fields. The average for each replicate from the individual plants was calculated for the analysis.

Experiment 2 - Effect of exogenous P and genotypes

The second experiment was conducted in a completely randomized design in a 3 x 6 factorial design with the same *S. lycopersicum* genotypes (*Wt*, *Nr*, and *notabilis*) and six P concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1 mM) with nine replicates ($n = 162$). $(\text{NH}_4)_3\text{PO}_4$ was used as the source of P. We used the same procedure from experiment 1 where two drops of each solution were placed over the slide and then leaf epidermal sections were transferred to these drops, covered with coverslips, and let to react at room temperature (22 ± 2 °C) for 10 min being then immediately photographed. Two leaves per plant were analyzed, with one slide per leaf and three fields per slide, totaling 972 sample fields. The average for each replicate of individual samples was calculated for the analysis.

Stomatal analysis

To perform the stomatal analysis in this study, it was essential that the sections were thin enough to preserve the epidermis, and that the epidermis was as well preserved as possible. Traditional paradermal sections did not work because the leaves of these genotypes are very thin, and their epidermis contains trichomes. In addition, the material could not be fixed because the objective was to verify stomatal movement. On this basis, and utilizing insights from previous tests, we developed a methodology in which the leaves were abraded from the adaxial to the abaxial face by wet scraping of the tissue layers. For this purpose, a steel blade was positioned perpendicular to the leaf, and a slight pressure of the steel blade was manually applied on the adaxial face of the leaflet. With movements in the direction from the base to the apex, scraping was performed, dissociating the leaflet tissues until intact fragments of the epidermis of the abaxial face were obtained. The leaflets were positioned on a stable flat surface for this scraping, using the bottom of a Petri dish as the flat surface. Before scraping, the material was covered with distilled water, and more water was added periodically to ensure hydration of the stomata so that they remained open and to facilitate the removal of the scraped tissue layers and the visualization of the remaining layers until only the facial epidermis was removed. An abaxial surface was observed (as a thin, semitransparent layer) (Fig. 1). The abaxial surface was selected because previous tests had revealed that the leaves of these selected genotypes have too few stomata in the adaxial surface, and the previous works reported that

this density can be as low as one stomata per mm^2 in specific tomato genotypes (Gay and Hurd, 1975).

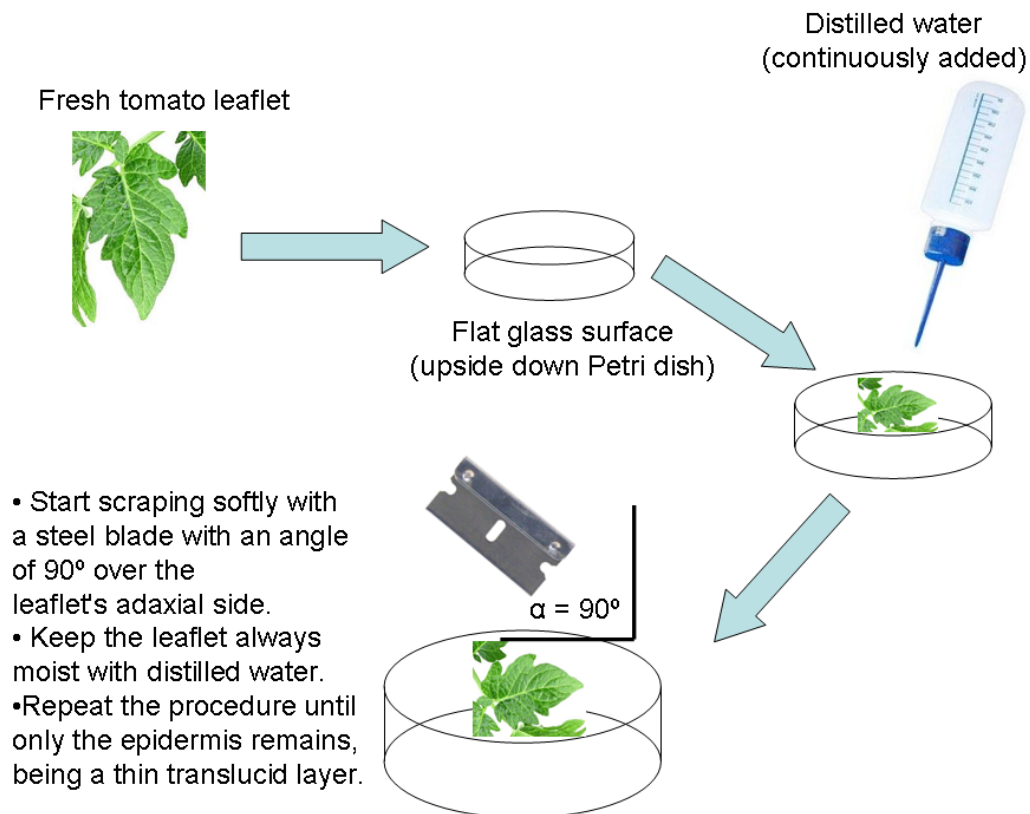


Figure 1. A scheme showing the overall method to obtain epidermal fragments.

The epidermal sections were added to a glass slide containing solutions of distilled water, ABA, or P as described for experiments 1 and 2 covered with coverslips and then let react for 10 min being immediately photographed after this period. Thus, the epidermal fragments were exposed just once to the solutions for 10 min which revealed sufficient to promote results according to previous tests. The ABA was prepared as a 10 mM solution in 0.1 molar potassium phosphate buffer at pH 6.5. The P concentration gradient from 0 to 1 mM was determined using dilutions of ammonium phosphate stock solution ($\text{NH}_4\text{H}_2\text{PO}_4$) in distilled water. The epidermal sections were not clarified or stained to preserve cellular functions and, in turn, the stomatal movement. The fragments were photographed using a digital camera attached to a Zeiss Axio Scope.A1 microscope (Zeiss, Oberkochen, Germany). The Fig. 2 shows the overall aspect of open and closed stomata using this method. Stomata were considered closed when the stomatal pore showed no light passing through and appeared as a solid dark line (Fig. 2B). Stomata were considered open when light passed through its stomatal pore showing a white ellipse-shaped region on the center (Fig. 2A).

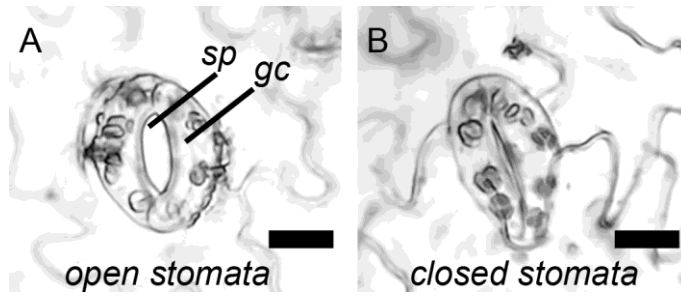


Figure 2. Example of open (A) and closed (B) stomata from leaf fragments of *S. lycopersicum*. sp = stomatal pore, gc = guard cells. Bars = 10 μm .

The stomatal variables evaluated were stomatal density, number of open and closed stomata, stomatal length and width, stomatal area, and stomatal pore area using the image analysis software ImageJ ver. 1.45s (Wayne Rasband National Institute of Health, USA). The following were also calculated: the proportion of pore area to the stomatal area using the equation $\%P = (\text{pore area}/\text{stomatal area}) \times 100$; the proportion of open and closed stomata with the equation $\%\text{closed} = (\text{number of closed stomata}/\text{total number of observed stomata}) \times 100$, and the percentage of open stomata was calculated as $\%\text{open} = 100 - \%\text{closed}$. Stomatal density was expressed in stomata per mm^2 . The application of ABA was not expected to change the stomatal density of the genotypes because it was formed during leaf development. We measured this parameter to be certain that differences between genotypes were not too large that affect the stomatal size. The percentage of closed stomata and the stomatal pore area can be regarded as the most significant variables to show the effect of P in stomatal closing in this work. Thus, we calculate the mean variation in these two parameters promoted by P as follows: $\text{MV}(\%) = \Sigma((\text{PVP}-\text{PVC})/\text{PVC}) \times 100 / 5$ where MV is the mean variation of the parameter, PVP is the mean parameter value measured under P concentrations and PVC is the parameter value measured in the control (0 mM of P). We then calculate the mean effect of P application as the mean of all these effects, expressed by the sum of these results divided by five which is the number of treatments where P was applied. This calculation is based on the tolerance index proposed by Wilkins (1978) which estimates the negative effect of an environmental factor in the biomass.

Estimation of the stomatal conductance

We estimate the maximum stomatal conductance using a modified equation from that proposed by Parlange and Waggoner (1970) and modified by Franks and Beerling (2009):

$$g_{\max} = \frac{\frac{dw}{v} \cdot SD \cdot pa_{\max}}{pd + \frac{\pi}{2} \sqrt{pa_{\max}/\pi}}$$

where dw = diffusivity of water vapour at 25°C ($0.0000249 \text{ m}^2 \text{ s}^{-2}$) and v = molar volume of air ($0.0224 \text{ m}^3 \text{ mol}^{-1}$) which we consider as both constants, SD is stomatal density (stomata m^2), $pa_{\max}$ is the maximum stomatal pore area (m^2); however, we have replaced this parameter

in the equation by the measured stomatal pore area (converted to m^2) since it was expected to give more accurate results. In addition, pd is the stomatal pore depth (m) considered to be equivalent to the width of an inflated, fully turgid guard cell according to Franks and Beerling (2009). This approach was necessary because it is not possible to use a porometer or an infrared gas analyzer to measure the stomatal conductance directly in tiny epidermal fragments in a microscopy slide and these types of equipment should be used in experiments with entire plants in the future.

Statistical Analysis

The data were subjected to analysis of variance (two-way ANOVA), and the means were compared by the Scott–Knott test or regression analysis at 5% significance ($P < 0.05$) using Sisvar 5.0 software (Ferreira 2011).

Results

Statistics

The summarized results of the two-way ANOVA are presented in Table 1 and demonstrate that for most of the variables analyzed, there was no interaction between the factors studied, except for the data from experiment 1. The coefficient of variation ranged from 5.3% to 31.0%, and there was an effect of genotype in both experiments and in all variables studied, with $P < 0.0001$ for all variables, which is the software calculation limit. The data also show that there was a significant effect of ABA in experiment 1 and P in experiment 2 for all variables tested. The only exception was stomatal density in experiment 1, which did not show significant variation as a function of ABA application.

Effect of exogenous ABA and genotypes

The *notabilis* genotype showed a higher stomatal density compared to the *Wt* and *Nr* genotypes, the two of which did not show significant differences from each other for this variable (Figure 3A). The ABA solution did not promote significant effects on stomatal density, and there was no solution x genotype interaction for this variable (Table 1 and Figure 3A).

There was a significant interaction between genotype and application of ABA for stomatal closure (Table 1). Nevertheless, ABA promoted stomatal closure in all genotypes, generating a higher percentage of closed stomata (Figure 3B). In the control treatment with distilled water, the *Nr* genotype (low sensitivity to ethylene) showed the highest proportion of

closed stomata, followed by the *Wt* and *notabilis* (deficient in ABA synthesis), which showed the lowest means (Figure 3B). The application of ABA promoted the closure of more than 90% of the stomata in the *Wt* and *Nr*, while the *notabilis* genotype had 70% of its stomata closed, generating similar means among the three genotypes (Figure 3B). The analysis of the percentage of open stomata revealed similar results, with a significant interaction among the experimental factors (Table 1) and the reduction in this parameter in all the genotypes by the application of ABA (Figure 3C). It was also observed that under the control treatment, the genotypes showed significant differences, with the highest percentage of open stomata being in the *notabilis* genotype (deficient in ABA synthesis), followed by the *Wt*, and the least responsive being the *Nr* (low sensitivity to ethylene). However, the application of ABA reduced the number of open stomata to less than 10% in the *Wt* and *Nr* genotypes and approximately 20% in the *notabilis* genotype, with similar means (Figure 3C).

Table 1. Summarized ANOVA results for all variables analysed, including mean square values, *F* test results, and *P* values. CV% = coefficient of variation.

Variable	CV%	Mean square value	<i>F</i> test value	<i>P</i> value
Experiment 1				
Stomatal density (G)	16.4	202952.7	213.5	<0.0001
*Stomatal density (S)	16.4	184.9	0.2	0.6595
Open stomata (G)	28.2	13813.2	0.4	0.6727
Open stomata (S)	28.2	203136.1	90.4	<0.0001
Closed stomata (G)	22.0	13813.2	90.4	<0.0001
Closed stomata (S)	22.0	203136.1	1329.0	<0.0001
Experiment 2				
Open stomata (G)	25.7	140191.9	775.7	<0.0001
Open stomata (P)	25.7	13143.9	72.7	<0.0001
Closed stomata (G)	28.2	140191.6	775.7	<0.0001
Closed stomata (P)	28.2	13144.0	72.7	<0.0001
Stomatal length (G)	5.3	3463.3	1648.4	<0.0001
Stomatal length (P)	5.3	23.9	11.4	<0.0001
Stomatal width (G)	6.4	2505.7	1231.7	<0.0001

Stomatal width (P)	6.4	33.6	16.5	<0.0001
Stomatal area (G)	10.9	5130071.2	1980.2	<0.0001
Stomatal area (P)	10.9	27552.05	10.6	<0.0001
Stomatal pore area (G)	31.0	165116.3	2794.6	<0.0001
Stomatal pore area (P)	31.0	6287.4	106.4	<0.0001
Stomatal pore proportion (G)	22.1	2992.4	2868.2	<0.0001
Stomatal pore proportion (P)	22.1	123.2	118.1	<0.0001
Maximum stomatal conductance (G)	5.0	29.0	834.0	<0.0001
Maximum stomatal conductance (P)	5.0	0.5	15.3	<0.0001
Mean variation promoted by P in the proportion of closed stomata (G)	31.7	139.96	10.5	<0.0001
Mean variation promoted by P in the stomatal pore area (G)	39.3	1495.3	7.4	<0.001

The P value limit of the software was 0.0001, so results lower than this limit are given as $p < 0.0001$. (G) = Genotype (*Wild type*, *Never ripe* or *notabilis*); (S) Solution (Water or ABA); (P) = Phosphorus concentration (mM). All variables showed a significant interaction at $p < 0.05$, except for those indicated by * = significant interaction at $p < 0.1$.

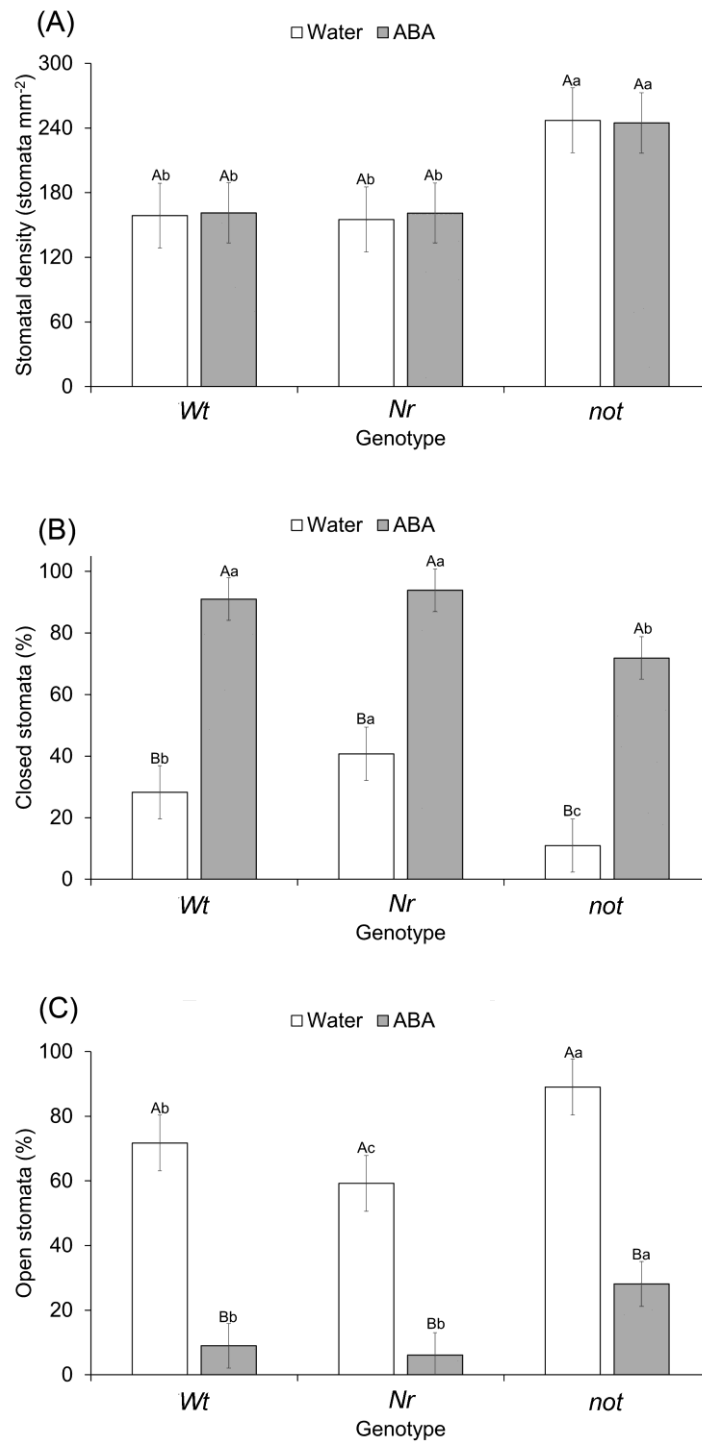


Figure 3. Density and stomatal opening of leaf fragments of *S. lycopersicum* exposed to water and ABA. (A) Stomatal density; (B) Percentage of closed stomata; (C) Percentage of open stomata. Means followed by the same letter do not differ according to Scott-Knott test for $P < 0.05$. Uppercase letters compare the solution type (water or ABA) and lowercase letters compare the genotypes (*Wt* - wild type, *Nr* - never ripe or *not* - notabilis). ($n = 54$)

Effect of exogenous phosphorus and genotypes

The percentage of closed stomata was approximately three times lower in the *notabilis* genotype (deficient in ABA production) than in the *Wt* and *Nr* (low sensitivity to ethylene), which showed similar means (Figure 4A). The application of P promoted the closure of stomata in all the genotypes evaluated, and this closure began at a concentration of 0.2 mM P, reaching maximum values close to 0.6 mM of the element in all the genotypes (Figures 4B, 4C, and 4D). The *Wt* genotype showed a highly significant increase in stomatal closure, with values increasing from 28.2% in the control to 66.3% at the highest concentration of P (Figure 4B). The *notabilis* genotype behaved similarly, with the number of closed stomata increasing from 10.9% in the control solution to 24.6% at the highest P concentration (Figure 4D). P had a smaller effect on the *Nr* genotype, and the percentage of closed stomata increased from 40.7% to 69.5% at the highest concentration of P (Figure 4C). Proportionally, these increases are equivalent to 134% in the *Wt* genotype, 125% in the *notabilis* genotype, and 70.1% in the *Nr* genotype.

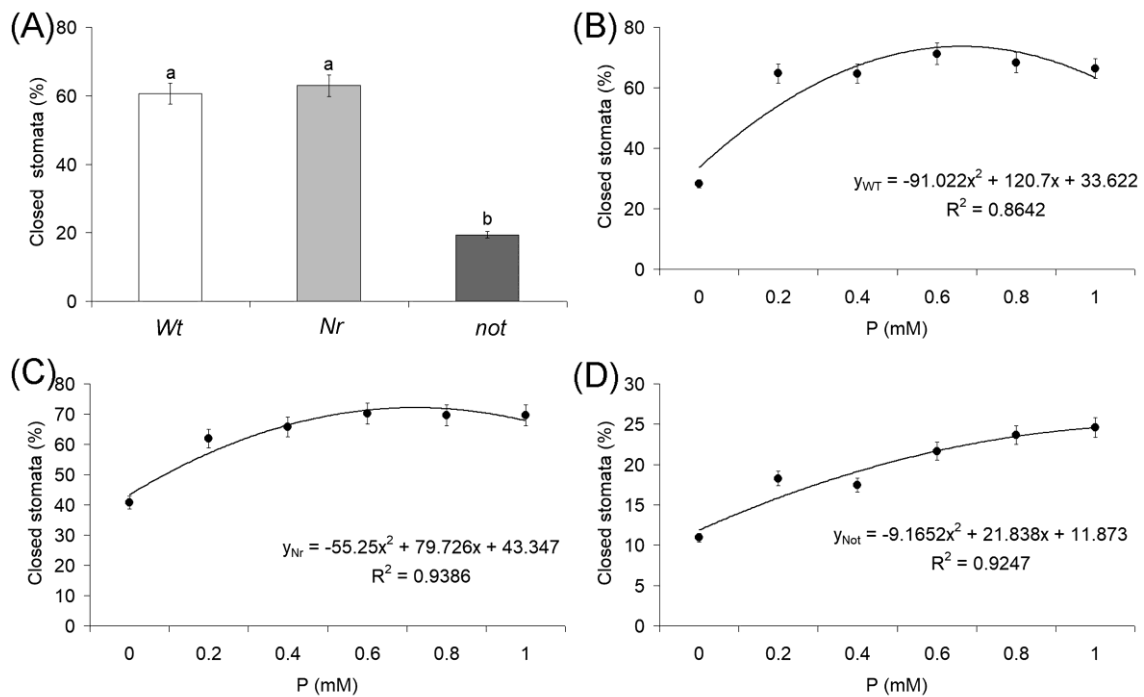


Figure 4. Percentage of closed stomata in leaves of *S. lycopersicum* genotypes exposed to different phosphorus (P) concentrations. (A) Comparison between genotypes; (B) effect of P on the *Wt* genotype; (C) effect of P on the *Nr* genotype (low sensitivity to ethylene); (D) effect of P on the *notabilis* genotype (deficient in ABA production). In the Figure A, means followed by the same letter do not differ according to Scott–Knott test for $P < 0.05$. For the figures B, C and D bars indicate the standard deviation, regression analysis $P < 0.05$. ($n = 162$)

The percentage of open stomata was higher in the *notabilis* genotype (deficient in ABA production) (Figure 5A). P application reduced the percentage of open stomata in all tested genotypes (Figures 5B, 5C, and 5D). The *Wt* genotype showed the greatest reduction in the percentage of open stomata, whereas the control treatment had 71.7% open stomata, and this value was reduced to 33.6% at the highest P concentration (Figure 5B). P promoted a smaller reduction in the percentage of open stomata in the *Nr* genotype, and this reduction decreased from 59.2% in the control treatment to 30.4% in the highest concentration of P (Figure 5C). The genotype least affected by P was *notabilis*, whose percentage of open stomata was reduced from 89.1% in the control to 75.4% at the highest concentration of P (Figure 5D).

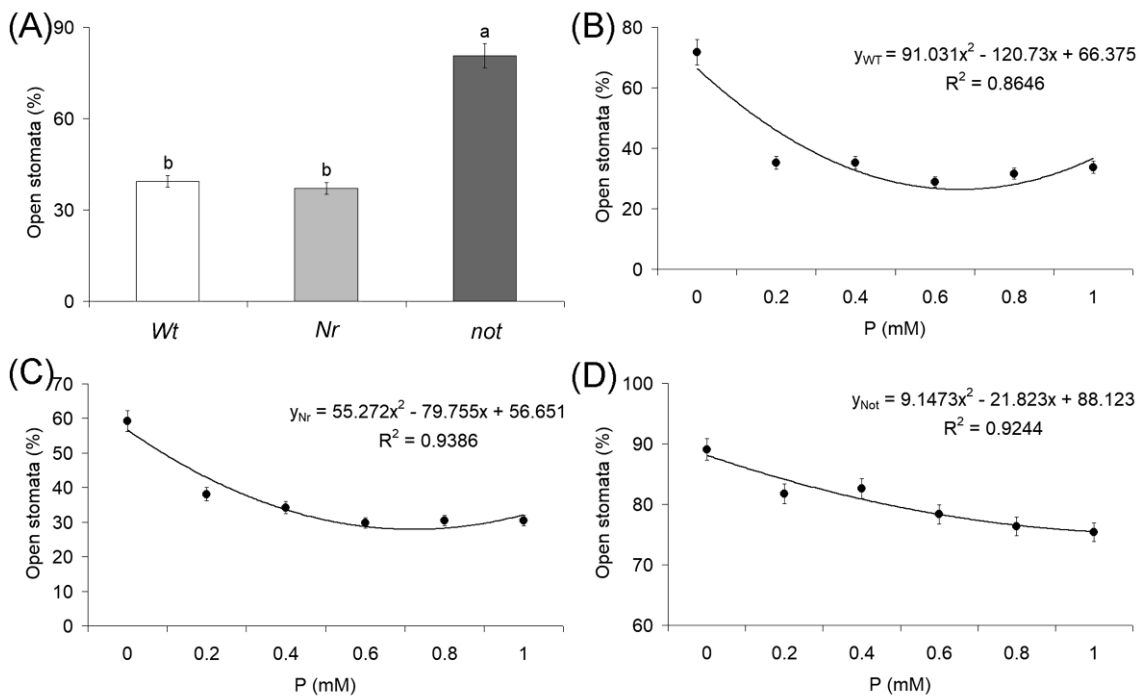


Figure 5. Percentage of open stomata in leaves of *S. lycopersicum* genotypes exposed to different phosphorus (P) concentrations. (A) Comparison between genotypes; (B) effect of P on the *Wt* genotype; (C) effect of P on the *Nr* genotype (low sensitivity to ethylene); (D) effect of P on the *notabilis* genotype (deficient in ABA production). In the Figure A, means followed by the same letter do not differ according to Scott–Knott test for $P < 0.05$. For the figures B, C and D bars indicate the standard deviation, regression analysis $P < 0.05$. ($n = 162$)

The stomatal length was greater in the *notabilis* genotype (deficient in ABA production), being 22% greater than that in the *Nr* genotype and 29% greater than that in the *Wt* genotype, which showed the shortest stomatal length (Figure 6A). P promoted stomatal elongation, increasing stomatal length in all the genotypes (Figures 6B, 6C, and 6D). The increase in stomatal length was 1.21 μm in the *Wt* genotype (Figure 6B), 1.29 μm in the *Nr* genotype (Figure 6C), and 1.13 μm in the *notabilis* genotype (Figure 6D). The stomatal width was also greater in the *notabilis* genotype than in the *Nr* and *Wt* genotypes (Figure 7A). P promoted a reduction in stomatal width in all the genotypes (Figures 7B, 7C, and 7D). The reduction in

stomatal width promoted by P was greater in the *notabilis* genotype, being reduced by 3.24 μm at the highest concentration of the element (Figure 7D); however, this reduction was only 0.32 μm in the *Wt* genotype (Figure 7B). The smallest reduction in stomatal width was observed in the *Nr* genotype, with a reduction of only 0.23 μm (Figure 7C).

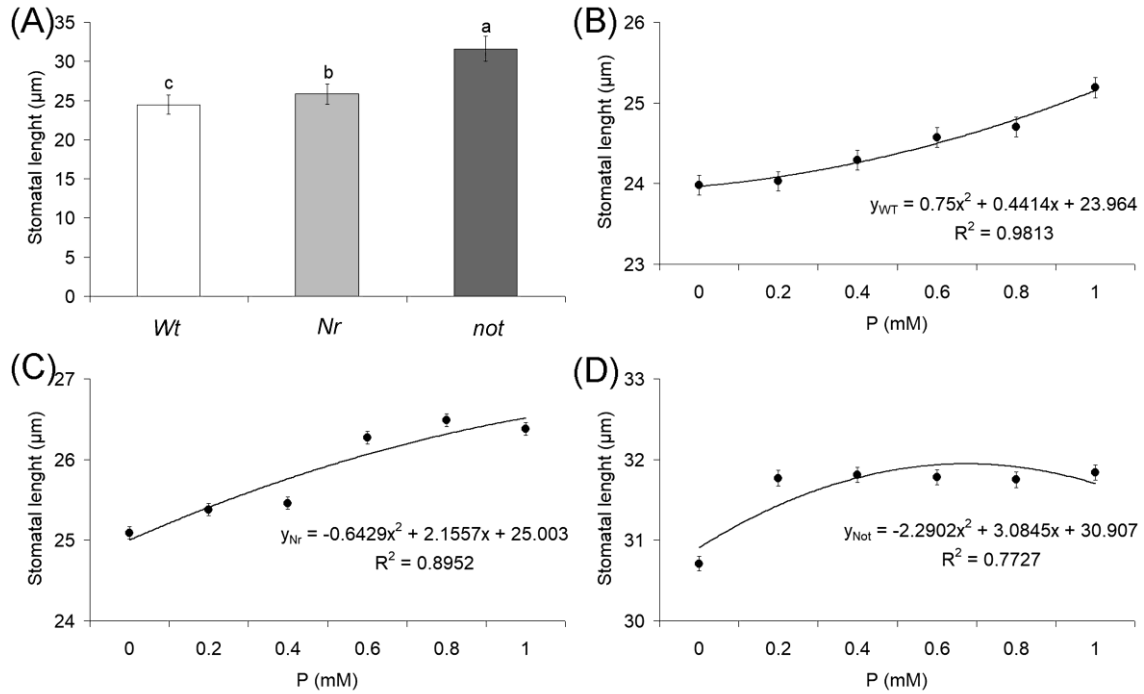


Figure 6. Stomatal length in leaves of *S. lycopersicum* genotypes at different phosphorus (P) concentrations. (A) Comparison between genotypes; (B) effect of P on the *Wt* genotype; (C) effect of P on the *Nr* genotype (low sensitivity to ethylene); (D) effect of P on the *notabilis* genotype (deficient in ABA production). In the Figure A, means followed by the same letter do not differ according to Scott–Knott test for $P < 0.05$. For the figures B, C and D bars indicate the standard deviation, regression analysis $P < 0.05$. ($n = 162$)

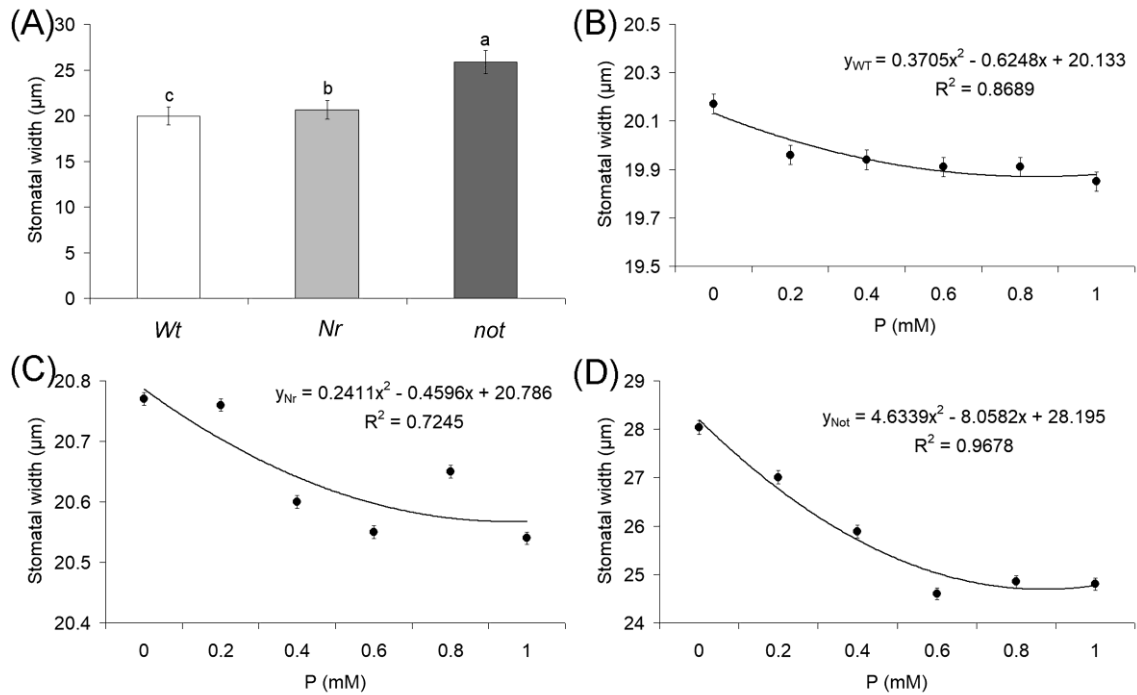


Figure 7. Stomatal width in leaves of *S. lycopersicum* genotypes with different phosphorus (P) concentrations. (A) Comparison between genotypes; (B) effect of P on the *Wt* genotype; (C) effect of P on the *Nr* genotype (low sensitivity to ethylene); (D) effect of P on the *notabilis* genotype (deficient in ABA production). In the Figure A, means followed by the same letter do not differ according to Scott–Knott test for $P < 0.05$. For the figures B, C and D, bars indicate the standard deviation, regression analysis $P < 0.05$. ($n = 162$)

The stomatal area was greater in the *notabilis* genotype (deficient in ABA production), whereas the *Nr* genotype (low sensitivity to ethylene) showed intermediate values, and the smallest areas were observed in the *Wt* genotype (Figure 8A). P application reduced the stomatal area of the three *S. lycopersicum* genotypes (Figures 8B, 8C, and 8D). Figure 8B demonstrates that the stomata of the *Wt* genotype reduced the stomatal area by approximately $25 \mu\text{m}^2$, approximately 6.5% of the total area. However, the *Nr* genotype was less responsive, with a reduction in area of approximately $10 \mu\text{m}^2$ or approximately only 2.5% of the total area (Figure 8C). The *notabilis* genotype, in turn, reduced approximately $90 \mu\text{m}^2$ or approximately 13.2% of the stomatal area, being the one that showed the most reduction in this parameter (Figure 8D).

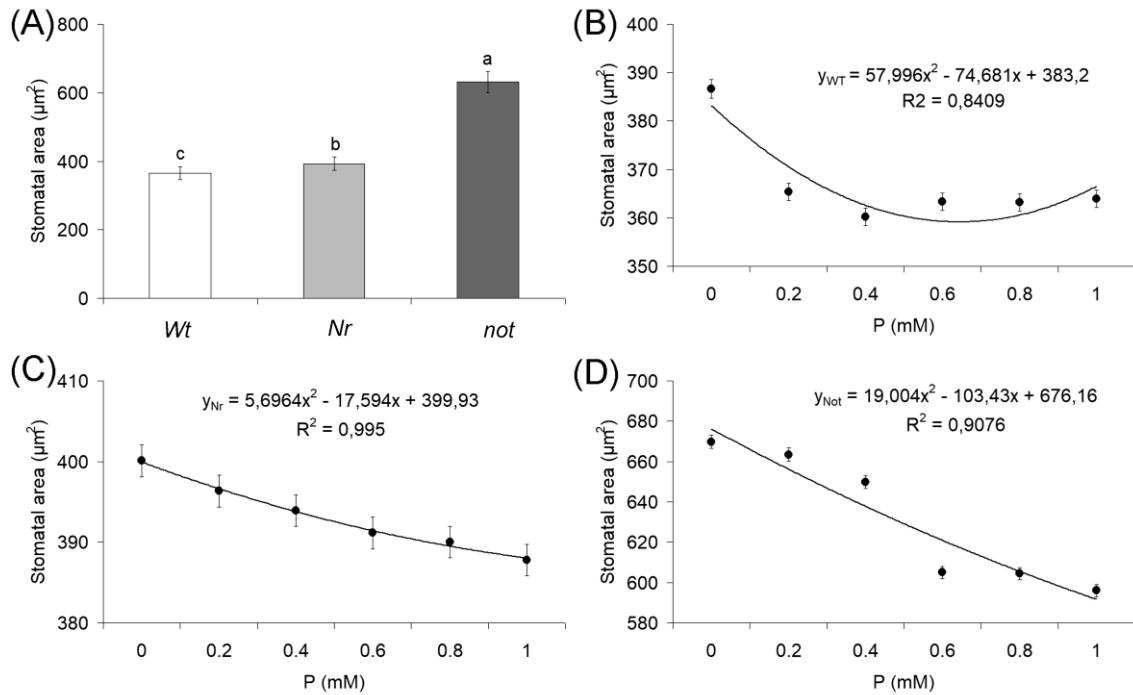


Figure 8. Stomatal area in leaves of *S. lycopersicum* genotypes at different phosphorus (P) concentrations. (A) Comparison between genotypes; (B) effect of P on the *Wt* genotype; (C) effect of P on the *Nr* genotype (low sensitivity to ethylene); (D) effect of P on the *notabilis* genotype (deficient in ABA production). In the Figure A, means followed by the same letter do not differ according to Scott–Knott test for $P < 0.05$. For the figures B, C and D bars indicate the standard deviation, regression analysis $P < 0.05$. ($n = 162$)

The stomatal pore area was also higher in the *notabilis* genotype (deficient in ABA production), and there were no significant differences between the *Nr* (low sensitivity to ethylene) and *Wt* genotypes (Figure 9A). The stomatal pore of the *notabilis* genotype was approximately five times larger than that of the other genotypes evaluated (Figure 9A). P application also reduced the pore area of all tested genotypes (Figures 9B, 9C, and 9D). P reduced the pore area in the *Wt* genotype by approximately 5 µm² or approximately 35.7% (Figure 9B). The reduction in the pore area of the *Nr* genotype was approximately 4 µm², or 30.7% (Figures 9C), and the greatest reduction was observed in the *notabilis* genotype, with a reduction of approximately 60 µm², equivalent to 67.7% of the pore area (Figure 9D). In the *Wt* and *Nr* genotypes, the reduction in pore opening was more marked in response to P concentrations of up to 0.6 mM, tending to stabilize at higher concentrations.

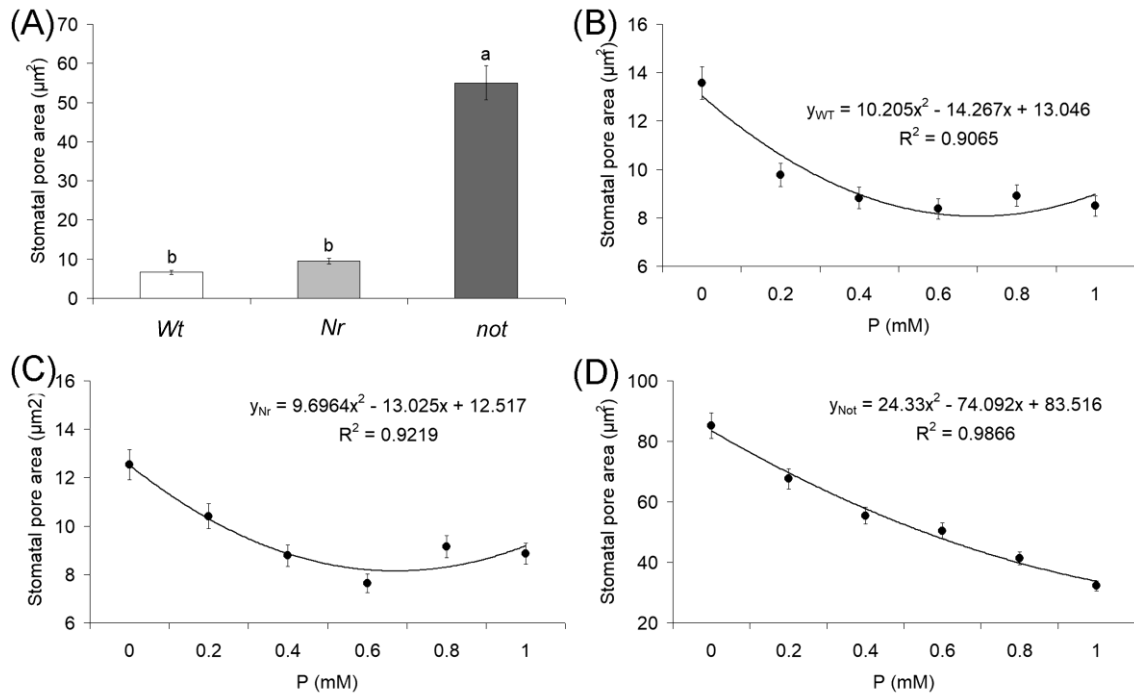


Figure 9. Stomatal pore area in leaves of *S. lycopersicum* genotypes with different phosphorus (P) concentrations. (A) Comparison between genotypes; (B) effect of P on the *Wt* genotype; (C) effect of P on the *Nr* genotype (low sensitivity to ethylene); (D) effect of P on the *notabilis* genotype (deficient in ABA production). In the Figure A, means followed by the same letter do not differ according to Scott–Knott test for $P < 0.05$. For the figures B, C and D bars indicate the standard deviation, regression analysis $P < 0.05$. ($n = 162$)

The proportion of the stomatal pore was higher in the *notabilis* genotype (deficient in the production of ABA). The *Wt* genotype showed intermediate values, and the lowest proportions were observed in the *Nr* genotype (low sensitivity to ethylene) (Figure 10A). The proportion of stomatal pore in the *notabilis* genotype was 3.5 times higher than that observed for *Wt* and *Nr* (Figure 10A). P reduced the proportion of stomatal pores in the three genotypes, and the effect was more pronounced in response to P concentrations of up to 0.6 mM (Figures 10B, 10C, and 10D). The pore proportion decreased by approximately 1% in the *Wt* and *Nr* genotypes (Figure 10B) and by approximately 6% in the *notabilis* genotype (Figure 10D) in response to a P concentration of 0.6 mM.

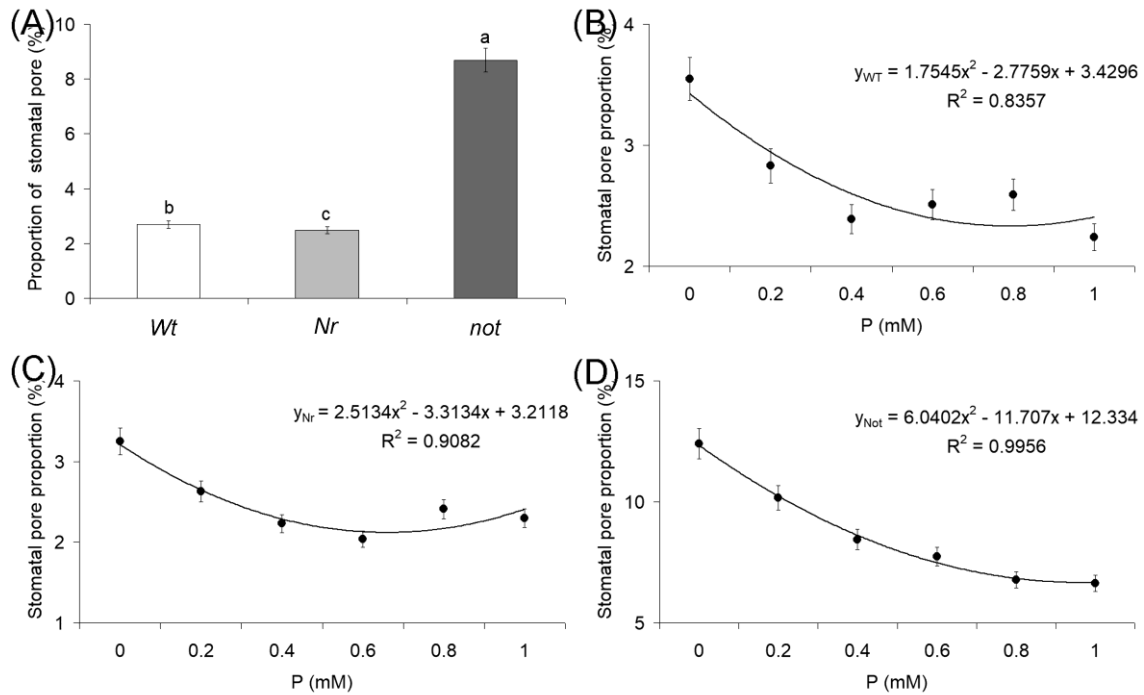


Figure 10. Stomatal pore proportion in leaves of *S. lycopersicum* genotypes at different phosphorus (P) concentrations. (A) Comparison between genotypes; (B) effect of P on the *Wt* genotype; (C) effect of P on the *Nr* genotype (low sensitivity to ethylene); (D) effect of P on the *notabilis* genotype (deficient in ABA production). In the Figure A, means followed by the same letter do not differ according to Scott–Knott test for $P < 0.05$. For the figures B, C and D bars indicate the standard deviation, regression analysis $P < 0.05$. ($n = 162$)

The stomatal conductance was 5.3 times higher in the *notabilis* genotype (deficient in ABA synthesis) as compared with *Nr* (low sensitivity to ethylene) and *Wt* which showed no significant differences between each other (Figure 11A). The increase in P concentration reduced the stomatal conductance in all genotypes with a linear fitting (Figures 11B, 11C, and 11D). The highest reduction in the stomatal conductance was observed in the *notabilis* genotype (Figure 11D) reduced by 41.9% followed by the *Wt* (33.9%) (Figure 11B) and *Nr* genotypes (31.9%) (Figure 11C). The *notabilis* genotype also showed the highest slope for all curves which was 6.9 times higher when compared with *Wt* (Figures 11B and 11D); the *Nr* genotype showed the lowest slope that was about 6% lower than *Wt* (Figures 11B and 11C).

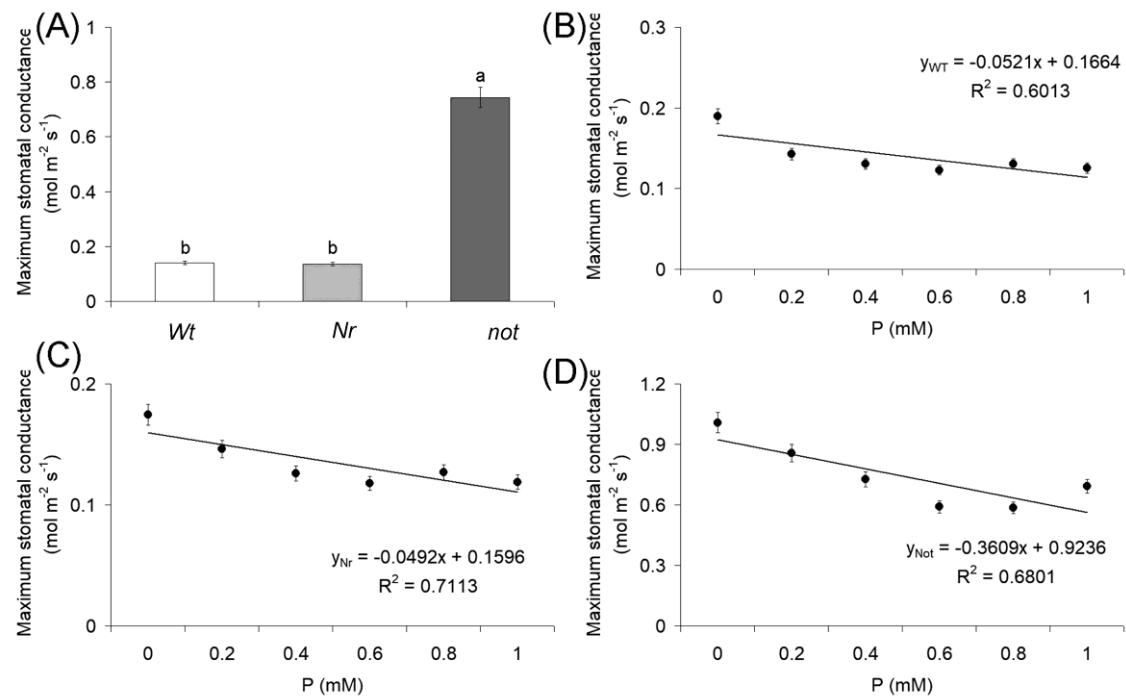


Figure 11. Maximum stomatal conductance in leaves of *S. lycopersicum* genotypes at different phosphorus (P) concentrations. (A) Comparison between genotypes; (B) effect of P on the *Wt* genotype; (C) effect of P on the *Nr* genotype (low sensitivity to ethylene); (D) effect of P on the *notabilis* genotype (deficient in ABA production). In the Figure A, means followed by the same letter do not differ according to Scott–Knott test for $P < 0.05$. For the figures B, C and D bars indicate the standard deviation, regression analysis $P < 0.05$. ($n = 162$)

P promoted different effects for each genotype regarding the mean variation for the proportion of closed stomata and stomatal pore area parameters (Figure 12). P promotes maximum effect in the proportion of closed stomata in the *notabilis* genotype, and the minimum effect was observed in *Nr* genotypes while the *Wt* genotype showed intermediate values (Figure 12A). In addition, P promoted lower variation in the stomatal pore area of the *Nr* genotype compared to *Wt* and *not* (Figure 12B).

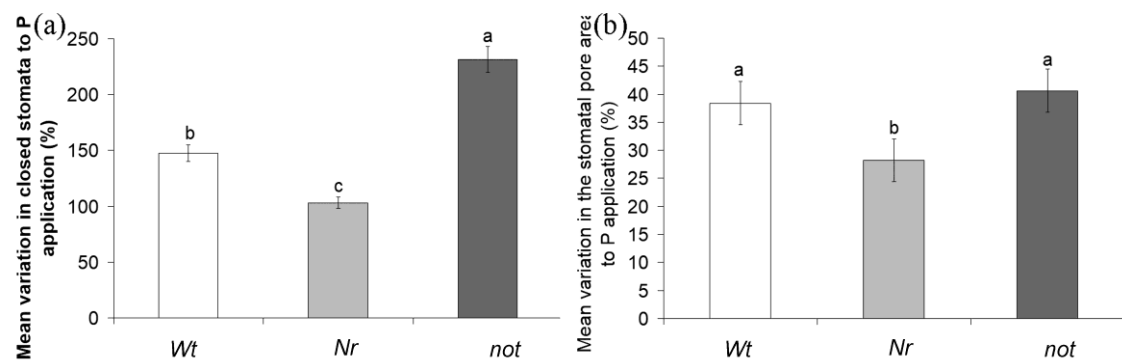


Figure 12. Mean variation in the proportion of closed stomata (a) and stomatal pore area (b) promoted by P application in leaves of *S. lycopersicum* genotypes. *Wt* genotype (wild type),

Nr genotype (low sensitivity to ethylene); and *notabilis* genotype (deficient in ABA production). Means followed by the same letter do not differ according to Scott–Knott test for $P < 0.05$. Bars = standard error. ($n = 118$)

Discussion

Effect of exogenous ABA application on the leaf epidermis to stomatal closure

The results of the experiment on the application of an ABA solution directly to epidermal fragments prepared with the methodology developed in this study were very positive. This first experiment was performed to verify the feasibility of testing the P solution directly on the epidermis since the methodology should allow the stomata to remain alive and responsive, closing when stimulated. ABA is the main phytohormone that signals stomatal closure under environmental stresses such as drought or infections (Bharath et al. 2021). The importance of ABA in this study is also related to its ability to interact with other phytohormones, such as ethylene (Agurla and Raghavendra 2016). The results of the present study indicate that the preparation of epidermal fragments by hydrated scraping technique was adequate, and the stomata remained functional, since all the studied *S. lycopersicum* genotypes closed their stomata when ABA was applied.

In this experiment, it was also observed that the responses to stomatal closure varied by genotype and that the *notabilis* genotype, which is deficient in ABA production, was less responsive. This genotype is deficient in the enzyme 9-cis-epoxycarotenoid dioxygenase (NCED), which is related to carotenoid cleavage and consequently promotes low production of ABA (Burbidge et al. 1999). As this genotype is deficient in ABA production, we expected that it might be less responsive to the hormone, but stomatal closure still occurred in response to the addition of exogenous ABA, indicating that this genotype still has ABA receptors that promote signaling for the closure of stomata. The signaling provided by ABA for stomatal closure is related to guard cell receptors, protein kinases, ion channels, and reactive oxygen species (Bharath et al. 2021). Therefore, the *notabilis* genotype remains responsive to the methodology applied in this study because it possesses receptors and signaling pathways, despite producing little endogenous ABA.

The *Nr* genotype responded to ABA application similarly to the *Wt*, closing the stomata and maintaining a similar percentage of closed stomata after exogenous application of the phytohormone. The *Nr* genotype has a deficiency in the *LeETR3* gene that encodes an ethylene receptor, thus exhibiting low sensitivity to this phytohormone and lower rates of response to ethylene, as evidenced by phenotypes such as low petal abscission (Carvalho et al. 2011). Because the *Nr* genotype is not especially responsive to ethylene and does not show limited responses to ABA, it behaved like the *Wt*, the control genotype, because the exogenous application of ABA promoted similar effects in these materials. The results, therefore, indicate that the methodology used, applying exogenous solutions that promote stomatal closure, is functional and that stomatal closure caused by ABA normally appears in the three genotypes

tested. This is an essential aspect for testing our hypothesis of the direct effect of P on stomatal closure, which was the study objective in the second experiment.

The stomatal density varied only as a function of the genotypes and not of the solution applied, which is an expected result. Stomata develop from protodermal cells and are among the first differentiated structures in the epidermis (Corrêa et al. 2017; Correa et al. 2022). The establishment of the stomatal density, therefore, occurs throughout leaf development and is fixed in a fully expanded leaf such as those collected for this experiment. The stomata in *S. lycopersicum* may vary regarding their position on the leaf axis (from the base to the apex) and even between the leaf faces (Gay and Hurd, 1975); therefore, some variation in the number of stomata between genotypes is expected. However, it is noteworthy that the *notabilis* genotype has a lower density compared to the *Nr* and *Wt* genotypes. We chose to evaluate only the stomata on the abaxial surface of the leaves because although there are stomata on the adaxial surface, according to Gay and Hurd (1975), the stomatal density on the adaxial surface can be as low as one stomata per mm². Notably, in this experiment, the stomatal density observed for the *S. lycopersicum* genotypes tested was similar to that of other genotypes of the species, ranging from 150 to 300 stomata per mm² (Fanourakis et al. 2015; Farber et al. 2016). Therefore, the first experiment demonstrated the feasibility of testing the exogenous application of a solution promoting stomatal closure to study the effect of P on this variable, which will be discussed in the following subsections.

Effect of exogenous phosphorus on the leaf epidermis to stomatal closure

Similar to what was observed with the application of exogenous ABA, the direct addition of P to the epidermis promoted stomatal closure in all genotypes; however, they demonstrated different levels of response to the nutrient. The *notabilis* genotype had a lower percentage of closed stomata when compared to the other genotypes, and this result may be related to the lower ABA production in these plants (Burbidge et al. 1999). The production of the ABA phytohormone is stimulated by many factors, with P deficiency among them (Battal et al. 2003; Nagatoshi et al. 2023), and can also be stimulated by an excess of the element (Cocozza et al. 2020). In the present experiment, stomatal closure was stimulated by increased P concentrations, especially starting at 0.6 mM P. However, as the *notabilis* genotype is deficient in ABA production, it is not reasonable to conclude that this closure was due to the production of this phytohormone, in addition, this genotype was the most responsive to P application. One of the main hypotheses of the present study is that P excess can stimulate ethylene production and that this phytohormone is responsible for stomatal closure and these results suggest that the case could explain stomatal closure in the ABA deficient *notabilis* genotype. Ethylene can directly promote stomatal closure by producing reactive oxygen species, especially H₂O₂, which signals internally in guard cells for closure (Desikan et al. 2006). Another result that underscores that the stomatal closure in the *notabilis* genotype was due to ethylene and not to ABA is the comparison of the results of the exogenous application of ABA with the application of P, since exogenous ABA promoted the closure of 80% of the stomata in this genotype, whereas P application closed a maximum of 25% of the stomata in this genotype. The addition of ABA directly promotes a much more marked effect in the

genotype deficient in its production, while P promotes an increase, but this is much smaller, possibly deriving from another signaling pathway. Therefore, the results indicate that stomatal closure in the *notabilis* genotype is probably related to ethylene and not ABA signaling, corroborating our hypothesis although no direct ethylene production was quantified and this should be addressed in future works.

The responses observed for the *Nr* genotype compared to the *notabilis* and *Wt* genotypes support the hypothesis that stomatal closure under excess P may be related to ethylene signaling. The previously reported result most consistent with this statement is that stomatal closure, represented by the percentage of closed stomata, was less pronounced in this genotype, which is little sensitive to ethylene (Carvalho et al. 2011). This genotype, as well as the others, showed stomatal closure by exogenous application of P in the epidermis; however, the *Nr* genotype showed a lower proportion of this effect suggesting a weaker effect from P. Although the final values for the percentage of closed stomata were similar between the *Nr* and *Wt* genotypes, the *Nr* genotype already had a greater number of closed stomata (41%) compared to only 28% in the *Wt* and 11% in the *notabilis*, whereas both the *Nr* and *Wt* genotypes exhibited approximately 70% of the stomata closed starting from P concentrations of 0.6 mM and *notabilis* about 25%. Therefore, both the *Wt* and the *notabilis* genotypes doubled the number of closed stomata by the application of P, while the *Nr* increased by slightly more than 50%, which characterizes a much lower response of this genotype. Because this genotype shows a deficiency in ethylene receptors (Hackett et al. 2000), many responses to this phytohormone are limited, such as those related to gene expression (Wilkinson et al. 1995) and H₂O₂ production, because this depends on ethylene signaling at the ETR1 receptor and is mediated by the NADPH oxidases encoded by *AtrbohF* (Desikan et al. 2006). Therefore, the reduction in the production of these *LeETR3* receptors in the *Nr* genotype leads to a low sensitivity to ethylene, which, although still present, was lower in intensity than levels in the *Wt* and *notabilis* genotypes. Future works regarding ethylene production using entire plants grown under excessive P may elucidate this topic further because such measurements are not possible in small epidermis fragments due to their limited biomass.

It is also important to discuss the difference in the responses of the *Nr* genotype when exogenous ABA was applied compared to P. This comparison shows that the response to exogenous ABA was much stronger than that of P in the *Nr* genotype, being comparable to what occurred with *Wt*. The application of ABA promoted a higher proportion of stomatal closure, reflected in the percentage of closed stomata (and inversely to that of open stomata), where approximately 40% of the closed stomata when water was applied increased to 80%, doubling the percentage of stomata. As already discussed, the effect was much smaller with the application of exogenous P, supporting the hypothesis that the signaling for stomatal closure occurs by the action of ethylene (since this genotype is less sensitive) and not via ABA.

The reduction in stomatal pore parameters in all the genotypes also corroborates the effect of P on the closure of these structures. The stomatal pore area is directly related to transpiration, and stomatal conductance increases with this parameter, as does the water loss by the plant (Kaiser 2009). In addition, the reduction in the estimated maximum stomatal conductance (Figure 9) supports that the reduction in the stomatal pore reduced the stomatal conductance because this parameter influences the stomatal conductance (Parlange and Waggoner 1970; Franks and Beerling 2009). Therefore, controlling the pore area is essential

for the plant to survive in the terrestrial environment, enabling it to control its water loss. This aspect of stomatal behavior is minimally functional in the *notabilis* genotype because this genotype is deficient in the production of ABA and does not exhibit such control. With the closure of the stomata, the area and proportion of the stomatal pore decreases, and as the *notabilis* genotype does not show this behavior, these parameters are much higher in this genotype than in the *Wt* and *Nr*. The addition of P, however, reduced the area and proportion of the stomatal pore in all the genotypes and the *notabilis* genotype stands out as the one that showed the greatest variation in this parameter as well as in the reduction of the stomatal conductance. Deficiency in ABA production in *S. lycopersicum* genotypes promotes excessive transpiration and water loss compared to *Wt* genotypes (Poór et al. 2019), and this water loss may be related to larger stomatal pore area, proportion, and stomatal conductance in the *notabilis* genotype. Due to this large pore size, the *notabilis* genotype showed the greatest reduction in pore area, proportion, and stomatal conductance compared to the *Nr* and *Wt* genotypes. This greater response of the *notabilis* genotype to stomatal pore closure also supports the hypothesis that ethylene production occurs by P excess since the *notabilis* genotype is deficient in the production of ABA.

The stomata of the three genotypes seem to have responded to exogenous P in a typical way regarding changes in length and width. Few studies demonstrate the changes in these two stomatal parameters during the stomatal closure process, but studies such as Desikan et al. (2006) demonstrated a linear reduction (in μm) in the stomatal opening that is related to the closing of the structures. For the stomata to open, an increase in the turgor pressure in the guard cells is needed, generated by the accumulation of solutes in the cytoplasm of these cells and the absorption of water by the cells and tissues around the stomata (Buckley et al. 2003). This increase in guard cell turgor promotes longitudinal elongation of the guard cells, which presses the apices and reduces the length of the stomata. With closure and loss of turgor pressure (Buckley et al. 2003), the stomatal width is reduced, and it can be expected that it will elongate during the process because there is a reduction in pressure between the apices of the two parallel guard cells. Therefore, the stomatal morphology of the *S. lycopersicum* genotypes responded as expected during closure, showing that the stomata were functional. It is also noteworthy that *notabilis* was the genotype that had the most markedly reduced stomatal width, supporting the hypothesis that the stomatal closure caused by P is due to ethylene regulation and not to ABA.

Relationship of phosphorus with ethylene production and stomatal closure

P is a macronutrient recognized as important as a structural component of biomolecules such as DNA, RNA, phospholipids, and phosphate carbohydrates, in addition to being essential for energy metabolism because it is a component of the molecules of ATP and ADP. However, P excess can cause a reduction in stomatal conductance and photosynthesis in some species, such as *Typha domingensis* Pers. (Santos et al. 2015) and as shown here in *S. lycopersicum* mutants. In addition, P excess can cause less development of the root system (Santos et al. 2021), and in *S. lycopersicum*, there are estimates that P can be toxic when it exceeds 1% of the

dry mass (Jones 1998). However, there has been no explanation reported for how P excess reduces stomatal conductance. Our main hypothesis is that P excess produces ethylene and that ethylene signals stomatal closure, leading to lower stomatal conductance, photosynthesis, and growth. The results for stomatal closure and stomatal conductance for the *Nr* and *notabilis* genotypes previously discussed support this hypothesis. P excess promotes ethylene production and negative growth and development responses in *Arabidopsis thaliana* L. (Shukla et al. 2017). Ethylene is known to be a phytohormone that can promote stomatal closure through the production of H₂O₂ (Desikan et al. 2006). Therefore, the results of the present study indicate that P excess stimulates ethylene synthesis in *S. lycopersicum*, which signals stomatal closure when exogenously applied to the epidermis.

Conclusion

The excessive P levels (0.6 mM or higher) trigger the stomatal closing when applied directly to leaf epidermis fragments *S. lycopersicum* reducing the stomatal conductance. Results indicate that phosphorus-triggered stomatal closing is mediated by ethylene that is produced under excessive P concentrations although future works with direct quantification of ethylene can further elucidate this subject.

Author Contributions

MPP: running the experiments, obtaining and analyzing the data and writing the first draft of the manuscript; CHGR: running the experiments and analyzing the data; PNS: running the experiments and analyzing the data; EMC revising the manuscript; RBK providing plant materials and revising the manuscript; JEL providing plant materials and revising the manuscript; FJP: advising the first author, conceptualizing, acquiring resources, designing and running the experiments, analyzing the data, curating the data, administering the project and writing the manuscript.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

Authors declare no conflict of interest regarding this work.

References

- Agurla S, Raghavendra AS (2016) Convergence and Divergence of Signaling Events in Guard Cells during Stomatal Closure by Plant Hormones or Microbial Elicitors. *Front Plant Sci* 7: 1332. <https://doi.org/10.3389/fpls.2016.01332>
- Battal P, Turker M, Tileklioğlu B (2003) Effects of different mineral nutrients on abscisic acid in maize (*Zea mays*). *Ann Bot Fenn* 40: 301–308. <http://www.jstor.org/stable/23726686>
- Bharath P, Gahir S, Raghavendra AS (2021) Absciscic Acid-Induced Stomatal Closure: An Important Component of Plant Defense Against Abiotic and Biotic Stress. *Front Plant Sci* 12. <https://doi.org/10.3389/fpls.2021.615114>
- Buckley TN, Mott KA, Farquhar GD (2003) A hydromechanical and biochemical model of stomatal conductance. *Plant Cell Environ* 26: 1767–1785. <https://doi.org/10.1046/j.1365-3040.2003.01094.x>
- Burbidge A, Grieve TM, Jackson A, Thompson A, McCarty DR, Taylor IB (1999) Characterization of the ABA deficient tomato mutant *notabilis* and its relationship with maize Vp14. *Plant J* 17: 427–431. <https://doi.org/10.1046/j.1365-313x.1999.00386.x>
- Campos-Soriano L, Bundó M, Bach-Pages M, Shiang S-F, Chiou T-J, Segundo BS (2020) Phosphate excess increases susceptibility to pathogen infection in rice. *Mol Plant Pathol* 21: 555–570. <https://doi.org/10.1111/mpp.12916>
- Carvalho RF, Campos ML, Pino LE, Lombardi-Crestana SL, Zsogon A, Lima JE, Benedito VA, Peres LEP (2011) Convergence of developmental mutants into a single tomato model system: Micro-Tom as an effective toolkit for plant development research. *Plant Methods* 7: 18. <https://doi.org/10.1186/1746-4811-7-18>
- Cocozza C, Brilli F, Pignattelli S, Pollastri S, Brunetti C, Gonnelli C, Tognetti R, Centritto M, Loreto F (2020) The excess of phosphorus in soil reduces physiological performances over time but enhances prompt recovery of salt-stressed *Arundo donax* plants. *Plant Physiol Biochem* 151: 556–565. <https://doi.org/10.1016/j.plaphy.2020.04.011>
- Corrêa FF, Pereira MP, Kloss RB, Castro EM, Pereira FJ (2017) Leaf ontogeny and meristem activity of *Typha domingensis* Pers. (Typhaceae) under different phosphate concentrations. *Aquat Bot* 136: 43–51. <http://dx.doi.org/10.1016/j.aquabot.2016.09.007>

Correa FF, Pereira MP, Kloss RB, Castro EM, Oliveira JPV, Pereira FJ (2022) Early leaf development of *Typha domingensis* Pers. (Typhaceae). *Botany Letters* 170: 247-257.

<https://doi.org/10.1080/23818107.2022.2136238>

Desikan R, Last K, Harrett-Williams R, Tagliavia C, Harter K, Hooley R, Hancock JT, Neill SJ (2006). Ethylene-induced stomatal closure in *Arabidopsis* occurs via AtrbohF-mediated hydrogen peroxide synthesis. *Plant J* 4: 907-916. <https://doi.org/10.1111/j.1365-313x.2006.02842.x>

Fanourakis D, Giday H, Milla R, Pieruschka R, Kjaer KH, Bolger M, Vasilevski A, Nunes-Nesi A, Fiorani F, Ottosen C (2015) Pore size regulates operating stomatal conductance, while stomatal densities drive the partitioning of conductance between leaf sides. *Annal Bot* 115: 555–565. <https://doi.org/10.1093/aob/mcu247>

Farber M, Attia Z, Weiss D (2016) Cytokinin activity increases stomatal density and transpiration rate in tomato, *J Exp Bot* 67: 6351–6362. <https://doi.org/10.1093/jxb/erw398>

Franks PJ, Beerling DJ (2009) Maximum leaf conductance driven by CO₂ effects on stomatal size and density over geologic time. *PNAS* 106: 10343-10347.

<https://doi.org/10.1073/pnas.0904209106>

Ferreira DF (2011) Sisvar: a computer statistical analysis system. *Cien Agrotec* 35: 1039–1042. <http://dx.doi.org/10.1590/S1413-70542011000600001>

Gay AP, Hurd RG (1975) The influence of light on stomatal density in the tomato. *New Phytol* 75: 37-46. <https://doi.org/10.1111/j.1469-8137.1975.tb01368.x>

Gruber BD, Giehl RFH, Friedel S, Von Wirén N (2013) Plasticity of the *Arabidopsis* root system under nutrient deficiencies. *Plant Physiology* 163, 161-179.

<https://doi.org/10.1104/pp.113.218453>

Hackett RM, Ho CW, Lin ZF, Foote HCC, Fray RG, Grierson D (2000) Antisense inhibition of the Nr gene restores normal ripening to the tomato Never-ripe mutant, consistent with the ethylene receptor-inhibition model. *Plant Physiol* 124: 1079-1086.

<https://doi.org/10.1104%2Fpp.124.3.1079>

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Berkeley: University of California.

Jones JB (1998) Phosphorus toxicity in tomato plants: when and how does it occur? *Commun Soil Sci Plant Anal* 29: 1779-1784. <https://doi.org/10.1080/00103629809370068>

Kaiser H (2009) The relation between stomatal aperture and gas exchange under consideration of pore geometry and diffusional resistance in the mesophyll. *Plant Cell Environ* 32: 1091-1098. <https://doi.org/10.1111/j.1365-3040.2009.01990.x>

- Kayoumu M, Iqbal A, Muhammad N, Li X, Li L, Wang X, Gui H, Qi Q, Ruan S, Guo R, Zhang X, Song M, Dong Q (2023) Phosphorus Availability Affects the Photosynthesis and Antioxidant System of Contrasting Low-P-Tolerant Cotton Genotypes. *Antioxidants* 12: 466. <https://doi.org/10.3390/antiox12020466>
- Liu D (2021) Root developmental responses to phosphorus nutrition. *J Integr Plant Biol* 63: 1065-1090. <https://doi.org/10.1111/jipb.13090>
- Nadeem F, Abbas S, Waseem F, Ali N, Mahmood R, Bibi S, Deng L, Wang R, Zhong Y, Li X (2024) Phosphorus (P) and Zinc (Zn) nutrition constraints: A perspective of linking soil application with plant regulations. *Environ Exp Bot* 226: 105875. <https://doi.org/10.1016/j.envexpbot.2024.105875>
- Nagatoshi Y, Ikazaki K, Kobayashi Y, Mizuno N, Sugita R, Takebayashi Y, Kojima M, Sakakibara H, Kobayashi NI, Tanoi K, Fujii K, Baba J, Ogiso-Tanaka E, Ishimoto M, Yasui Y, Oya T, Fujita Y (2023) Phosphate starvation response precedes abscisic acid response under progressive mild drought in plants. *Nature Commun* 14: 5047. <https://doi.org/10.1038/s41467-023-40773-1>
- Parlange JY, Waggoner PE (1970) Stomatal Dimensions and Resistance to Diffusion. *Plant Physiol* 46: 337-342. <https://doi.org/10.1104/pp.46.2.337>
- Poór P, Borbély P, Czékus Z, Takács Z, Ördög A, Popović B., Tari I (2019) Comparison of changes in water status and photosynthetic parameters in wild type and abscisic acid-deficient *Solanum lycopersicum* cv. Rheinlands Ruhm exposed to sublethal and lethal salt stress. *J Plant Physiol* 232: 130-140. <https://doi.org/10.1016/j.jplph.2018.11.015>
- Raghothama KG, Karthikeyan AS (2005) Phosphate acquisition. *Plant Soil* 274: 37-49. <https://doi.org/10.1007/s11104-004-2005-6>
- Santos JCB, Castro EM, Duarte VP, Pereira MP, Correa FF, Pereira FJ (2021) Root growth and anatomy of *Typha domingensis* Pers. related to phosphorus availability. *Acta Limnol Bras* 33. <http://dx.doi.org/10.1590/s2179-975x2120>
- Santos KR, Pereira MP, Ferreira ACG, Rodrigues LCA, Castro EM, Corrêa FF, Pereira, FJ (2015) *Typha domingensis* Pers. responses to leaf anatomy and photosynthesis as influenced by phosphorus. *Aquatic Bot* 122: 47–53. <http://dx.doi.org/10.1016/j.aquabot.2015.01.007>
- Shukla D, Rinehart CA, Sahi SV (2017) Comprehensive study of excess phosphate response reveals ethylene mediated signaling that negatively regulates plant growth and development. *Scientific Reports* 7: 3074. <https://doi.org/10.1038/s41598-017-03061-9>
- Wilkins DA (1978) The measurement of tolerance to edaphic factors by means of root growth. *New Phytol* 80: 623–633. <https://doi.org/10.1111/j.1469-8137.1978.tb01595.x>

Wilkinson JQ, Lanahan MB, Yen H, Giovannoni JJ, Klee HJ. (1995) An ethylene-inducible component of signal transduction encoded by never-ripe. *Science*, 270/5243, 1807-1809.

<https://doi.org/10.1126/science.270.5243.1807>

Wurzburger N, Bellenger JP, Kraepiel AML, Hedin LO (2012) Molybdenum and phosphorus interact to constrain asymbiotic nitrogen fixation in tropical forests. *PLoS ONE* 7(3): e33710.

<https://doi.org/10.1371/journal.pone.0033710>

Zakrzewska J, Mitrović AL, Mutavdžić D, Dučić T, Radotić K (2020) Phosphorus homeostasis in *Populus alba* L. under excess phosphate conditions, assessed by ³¹P nuclear magnetic resonance spectroscopy and X-ray microfluorescence. *Environ Sci and Pollut Res* 27: 3320–3328. <https://doi.org/10.1007/s11356-019-07200-9>

ARTIGO 2 - Excessive P reduces the photosynthesis and growth in *Solanum lycopersicum* by stomatal closing mediated by ethylene and ABA

Artigo submetido e elaborado de acordo com as normas do periódico Protoplasma

Excessive P reduces the photosynthesis and growth in *Solanum lycopersicum* by stomatal closing mediated by ethylene and ABA

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Statements and declarations

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author, Fabricio José Pereira, upon request.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflicts of interest.

Abstract

Phosphorus (P) is an essential macronutrient for plant growth and development, however, both its deficiency and excess can be harmful. Although the effects of excess P are still poorly understood, research has shown that plants exposed to excessive levels of P exhibit reductions in stomatal conductance, photosynthesis, and growth. The aim of this study was to investigate the effect of different P concentrations on stomatal responses, photochemical parameters, growth, and development of three *Solanum lycopersicum* genotypes: *wild type*, *Never ripe* (lower sensitivity to ethylene), and *Notabilis* (deficient in ABA production). The plants were grown in a growth room, and stomatal parameters, photochemical parameters, stomatal conductance, transpiration, and H₂O₂ content in the leaves were analyzed. Excessive P concentrations promoted stomatal closure, reduced stomatal conductance and transpiration but did not cause significant changes in photochemical parameters in *S. lycopersicum*, indicating that the negative effects of excess P may be related to limitations in CO₂ acquisition. Furthermore, excess P increased leaf H₂O₂ content. The absence of negative effects of excess P in the *Never ripe* and *Notabilis* genotypes suggests that stomatal closure related to excess P depends on ethylene and ABA signaling to guard cells.

Keywords: Absciscic acid, ethylene, guard cells, phosphorus, stomatal closure, tomato mutants.

Highlight: Excessive P stimulates ethylene and ABA signaling, closing stomata and limiting photosynthesis and growth in tomato.

1. Introduction

Phosphorus (P) is an essential nutrient for plant growth and development being part of the structure of DNA, RNA, ATP, and phospholipids among other molecules. Phosphorus limitation is well understood and there are several works reporting reductions in growth and photosynthesis under this condition (Chiera et al., 2002; Assuero et al., 2004; Gruber et al., 2013; Xue et al., 2017). P starvation limits leaf gas exchange, photosynthesis, photosynthetic efficiency, and nutrient uptake (Santos et al., 2015; Meng et al., 2021; Kayoumu et al., 2023). Excessive P causes eutrophication of aquatic environments (Sharpley and Tunney, 2000; Smith and Schindler, 2009) and reduces photosynthesis in macrophyte species (Santos et al., 2015). Thus, adequate P concentration is essential for plant development, and excessive P can even disturb Zn absorption, causing nutritional imbalance in plants (Zhang et al., 2012).

Examples of negative effects of excessive P levels in plants have been reported in recent publications, for instance, reduced growth and development of *Populus alba* L. under high P concentrations (Zakrzewska et al., 2020). Excessive P also reduced the meristematic activity and growth of *Arabidopsis thaliana* (L.) Heynh (Shukla et al., 2017). The aquatic plant *Typha domingensis* (Pers.), often considered favored by eutrophic environments, showed reduction of the palisade parenchyma, photosynthesis, stomatal conductance, mitotic activity in leaf meristems and root growth P concentrations of 0.6 mM or higher (Santos et al., 2015; Corrêa et al., 2017; Santos et al., 2021). Although negative effects from excessive P have been published, the mechanistic interpretation of their reasons is unclear. The lower photosynthesis and stomatal conductance promoted by excessive P are of particular relevance. According to Shukla et al. (2017), excessive P increases the ethylene production. Thus, one of the reasons for reduced stomatal conductance and photosynthesis under excessive P must be related to ethylene signaling. Ethylene is known for its role in signaling under environmental stress and also regulates the synthesis of abscisic acid (ABA) (Grossmann and Hansen, 2001). We hypothesize that under excessive P levels, the increased ethylene production may cause stomatal closing directly or intermediate by ABA, reducing stomatal conductance and photosynthesis.

In this work, we use *Solanum lycopersicum* L. cv. Micro-Tom (MT) mutants to investigate the excessive P because of its reduced size being easy to handle as a model and also because of its mutants that exhibit different ethylene and ABA responses. MT mutants show specific hormonal responses: the *notabilis* (*not*) shows lower ABA production and the *Never ripe* (*Nr*) have limited ethylene (Burbidge et al., 1999; Hackett et al., 2000; Carvalho et al., 2011) when compared with the wild type genotype (*WT*). Thus, *notabilis* and *Nr* genotypes are expected to be less responsive to excessive P compared to *WT* if its effects are related to ethylene or ABA.

Thus, this study aimed to investigate the effect of different P concentrations in the stomatal responses, photochemical parameters, growth, and development of *S. lycopersicum*.

2. Material and methods

2.1 Plant material

In this experiment, three genotypes of *Solanum lycopersicum* L. from the Micro-Tom (MT) variety were used: *Wild type* (WT), *Never ripe* (Nr), and *Notabilis* (not). The Nr genotype is defective in the ethylene receptor *LeETR3* and it expresses low sensitivity to ethylene, and the *notabilis* genotype is defective in the carotenoid-cleaving enzyme 9-cis-epoxy carotenoid dioxygenase and therefore defective in the production and accumulation of abscisic acid (ABA) (Carvalho et al., 2011). These genotypes were chosen to correlate the P effect with the ethylene signaling in roots (Nr) for ABA production (*notabilis*), which could transfer the signal to leaves closing the stomata.

2.2 Experimental design

The work was conducted at the Universidade Federal de Alfenas, Alfenas, Minas Gerais, Brazil. Seeds were sown in plastic pots of 300 ml containing 270 ml of sand and 90 ml of nutrient solution (Hoagland and Arnon, 1950). The water lost by evapotranspiration was measured gravimetrically and replaced daily. The plants were kept in a growth chamber at 25 ± 2 °C, 12 h photoperiod, 60% relative humidity, and 40 W m^{-2} of irradiance.

The experimental design was completely randomized in a 3 x 6 factorial scheme comprising three *S. lycopersicum* genotypes (WT, Nr, and *notabilis*) and six P concentrations (0, 0.2, 0.4, 0.6, 0.8, and 1 mM) with four replicates ($n = 72$). The P concentrations in the nutrient solution were changed according to Santos et al. (2021) and Table 1. The nutrient solution was replaced weekly. We covered each pot with a plastic cover to keep a water-saturated atmosphere inside the chamber. In previous experiments, excessive water loss from the *notabilis* genotype caused its mortality. We kept all genotypes under this cover.

Table 1. Concentrations of P and N sources in the nutrient solution

<i>P level</i> (mM)	<i>NH₄H₂PO₄</i> (mM)	<i>KNO₃</i> (mM)	<i>Ca(NO₃)₂</i> (mM)	<i>MgSO₄</i> (mM)	<i>Total N</i> (mM)
0	0	0.90	0.35	0.10	1.6
0.2	0.2	0.80	0.30	0.10	1.6
0.4	0.4	0.70	0.25	0.10	1.6
0.6	0.6	0.50	0.25	0.10	1.6
0.8	0.8	0.40	0.20	0.10	1.6

1	1	0.30	0.15	0.10	1.6
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2.3 Dry mass analysis

At the end of the experiment two plants per pot were collected and oven-dried at 60 °C for 72 h and then the dry mass was measured with an analytical scale ATX224R (Shimadzu, Tokyo, Japan).

2.4 Chlorophyll content and photochemical analysis

The chlorophyll content and photochemical analyses were performed on two fully developed leaves per plant with good phytosanitary conditions. These analyses were performed at the leaf basis, middle, and apex. The chlorophyll content was measured using a handheld chlorophyll meter SPAD-CHL Plus (AtLEAF, Wilmington, USA). The photochemical parameters were assessed with a modulated fluorometer Mini-PAM II (Heinz Walz, Effeltrich, Germany) using the leaf clip for the evaluations under light conditions, and for the dark evaluations leaves were kept in the dark for 30 min before measurements were taken. The following parameters were evaluated: the quantum yield from photosystem II (Φ PSII), the electron transfer rate (ETR), and the maximum yield from photosystem II (Fv/Fm).

2.5 Stomatal analysis

For the stomatal analysis, two leaflets from two completely developed leaves in good health were evaluated per plant (four leaflets per plant). The abaxial surface of *S. lycopersicum* leaves shows more stomata (Gay and Hurd, 1975). The abaxial surface was used to obtain paradermal sections from fresh leaflets, avoiding stomatal closure promoted by fixation. Paradermal sections were kept hydrated using distilled water during the entire process. Sections were performed using steel blades scraping the leaf from the adaxial to the abaxial surface until only the abaxial epidermis rested; the tissue was then transferred to a slide containing distilled water and covered with a coverslip. The slides were immediately photographed using an image capture system coupled to an Eclipse Si microscope (Nikon, Tokyo, Japão). The images were evaluated using the ImageJ 1.45s software (Wayne Rasband National Institute of Health, USA). The number of stomata per image was counted and the area of the image was measured (in

μm^2) to calculate the stomatal density (SD) as follows: $\text{SD} = [(\text{number of stomata per image} \cdot 10^6)/\text{image area}]$. The stomatal density was given in stomata per mm^2 . The number of open and closed stomata was counted per image and the percentage of open stomata (open%) was calculated as follows: $\text{open\%} = [(\text{number of open stomata per image}/\text{total stomata per image}) \cdot 100]$. The percentage of closed stomata (closed%) was calculated as follows: $\text{closed\%} = [(\text{number of closed stomata per image}/\text{total stomata per image}) \cdot 100]$.

2.6 Estimation of the stomatal conductance and transpiration

We estimate the maximum stomatal conductance using a modified equation from that proposed by Parlange and Waggoner (1970) and modified by Franks and Beerling (2009):

$$g_{\max} = \frac{\frac{dw}{v} \cdot \text{SD} \cdot pa_{\max}}{pd + \frac{\pi}{2} \sqrt{pa_{\max}/\pi}}$$

Where dw = diffusivity of water gaseous phase at 25°C ($0.0000249 \text{ m}^2 \text{ s}^{-2}$) and v = molar volume of air ($0.0224 \text{ m}^3 \text{ mol}^{-1}$) which we consider as both constants, SD is stomatal density (stomata m^2), $pa_{\max}$ is the maximum stomatal pore area (m^2); however, we have replaced this parameter in the equation by the measured stomatal pore area (converted to m^2) since it was expected to give more accurate results. In addition, pd is the stomatal pore depth (m) considered equivalent to the width of an inflated, fully turgid guard cell according to Franks and Beerling (2009).

The transpiration (E) was estimated using a gravimetric evaluation of water loss and the leaf area. The first fully developed leaf (with all leaflets) was collected from one plant per replicate. Leaves were immediately photographed after collection and the leaf area was measured with the ImageJ 1.45s software (Wayne Rasband National Institute of Health, EUA). Leaves were immediately weighed in an analytical scale ATX224R (Shimadzu, Tokyo, Japan), placed in open Petri dishes, and weighed again at 10-minute intervals for 30 min. The difference between the initial and final masses (dM) was calculated (in g) being considered the water loss. Then, we calculate the water loss in moles ($dM/18$) accepting that 1 g equals 1 ml of water and the water molecular weight (18 g); the water loss in moles was converted to millimoles by multiplying it by 10^3 . The leaf area (LA) was measured in cm^2 and converted to m^2 by dividing

it by 10^4 . The time elapsed (T_{seconds}) (30 min) was converted to seconds ($30 * 60 = 1800$ s). The transpiration (E) can be calculated as follows: $E = [(dM/18*1000)/(LA_{\text{cm}^2}/10000)]/T_{\text{seconds}}$

2.7 H₂O₂ quantification

We evaluate the H₂O₂ concentration in leaves because this molecule is related to ABA-induced stomatal closing (Zhang et al., 2001). For this analysis, fully developed and healthy leaves were collected at the end of the experiment and immediately frozen in liquid nitrogen, stored at -20 °C until the analysis. For the extraction, 200 mg of leaves were grounded in liquid nitrogen using a mortar and pestle. The extraction was performed using 2 ml of a extracting solution comprised of 1924 µl of phosphate buffer 100 mM and pH 7, 40 µl of MgCl₂.6H₂O 500 mM, 20 µl of EDTA 100 mM, 16 µl of Phenylmethylsulfonyl Fluoride (PMSF) 100 mM, 8 µl of dithiotreitol (DTT) 500 mM, and 20 mg of polivinilpolipirrolidona (PVPP). The extract was transferred to Eppendorf tubes and centrifuged at 12000 g for 20 min and -4 °C, the supernatant was collected and stored at -20 °C until analysis. The H₂O₂ is usually extracted with trichloroacetic acid (TCA) (Velikova et al., 2000; Alexieva et al., 2001); however, our plants were too small and could not collect large samples. We previously tested a protocol for general extraction from our laboratory used in antioxidant system analysis (Pereira et al., 2014; Pereira et al., 2017, Duarte et al., 2021) and compared it with TCA extraction with no significant alterations in the results. Thus, we decided to perform the extraction with the phosphate buffer as explained because it uses a lower amount of sample.

The quantification was adapted from Velikova et al. (2000) and Alexieva et al. (2001). For the H₂O₂ quantification, in essay tubes, 500 µl of the extract was added to 100 µl of TCA 0.1% (m V⁻¹), 400 µl of phosphate buffer 10 mM pH 7, and 1000 µl of KI 1.0 M. For the blank, the extraction buffer was used instead of the extract. A standard curve was built for the quantification replacing the extract by known H₂O₂ of 0 (distilled water), 1, 10, 50, and 100 mM of H₂O₂ in distilled water; the R² was 0.99 and the regression equation was $y = 0.0035x + 0.0054$. The samples were kept in the dark for 1 h for the reaction and after this period the H₂O₂ was quantified in a Halo SB-10 spectrophotometer (Dynamica Scientific Ltd, Livingston, UK) at 390 nm.

2.8 Statistical analysis

The data were subjected to analysis of variance (two-way ANOVA), and the means were compared by the Scott–Knott test or regression analysis at 5% significance ($P < 0.05$) using Sisvar 5.0 software (Ferreira, 2011).

3. Results

No significant differences for the dry mass between the genotypes were observed until the 0.4 mM of P; however, at P levels of 0.6 mM or higher the *Nr* (lower sensitivity to ethylene) genotype showed higher dry mass than the *WT* genotype and higher than the *notabilis* (defective in ABA production) genotype at the 0.6 and 0.8 mM (Fig. 1). The P concentrations of 0.6 mM or higher reduced the dry mass of the *WT* genotype whereas caused no significant effect in the *notabilis* genotype and increased the *Nr* dry mass at the 0.8 mM concentration (Fig. 1).

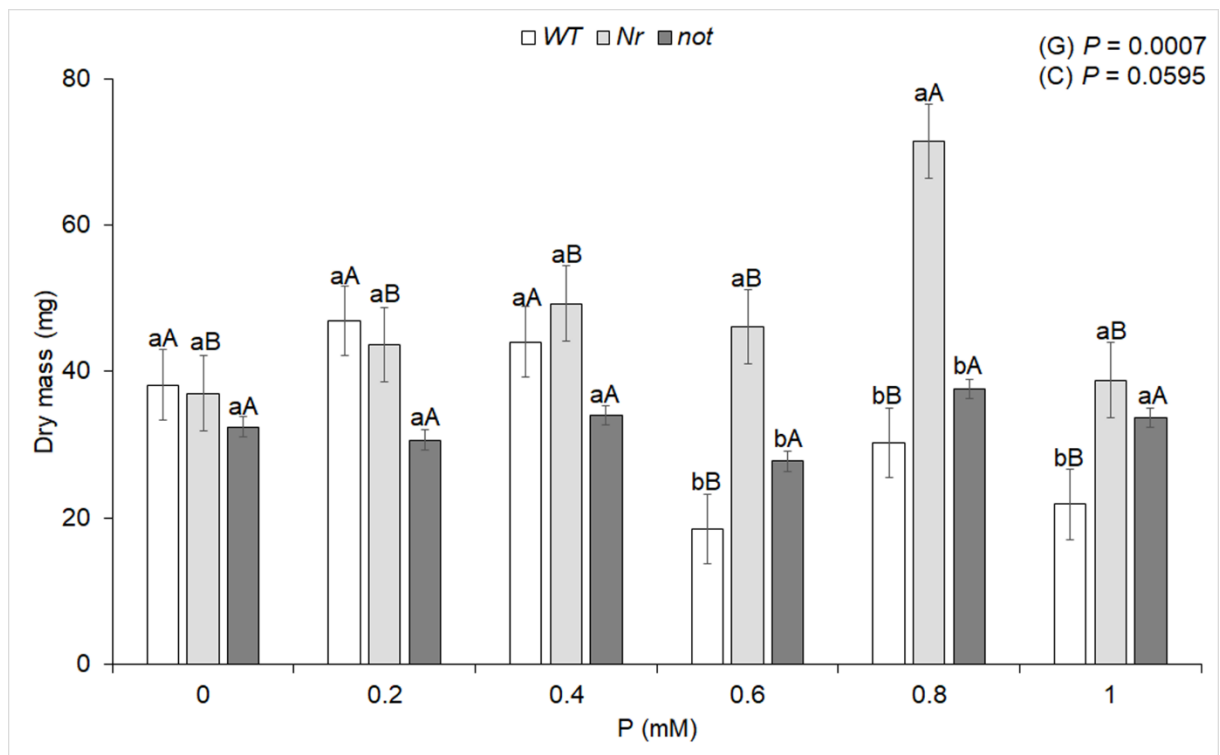


Fig. 1. Dry mass of *S. lycopersicum* under different phosphorus concentrations. Means followed by the same letter do not differ according to the Scott–Knott test for $P < 0.05$. Uppercase letters compare the solution type (water or ABA) and lowercase letters compare the genotypes [*WT* - wild type, *Nr* - *Never ripe* (lower sensitivity to ethylene) or *not* - *notabilis* (deficient in ABA production)]. G = genotypes, C = phosphorus concentrations ($n = 72$).

The P concentrations and *S. lycopersicum* genotypes did not modify the chlorophyll content (Fig. 2A and 2B). The increase of P concentrations increased the quantum yield of the

photosystem II (Φ PSII) and the electron transfer rate (ETR) of *S. lycopersicum* genotypes (Fig. 3B and D). The P concentrations and *S. lycopersicum* genotypes promoted no significant changes in the maximum yield of the photosystem II (Fv/Fm) (Fig. 3E and F).

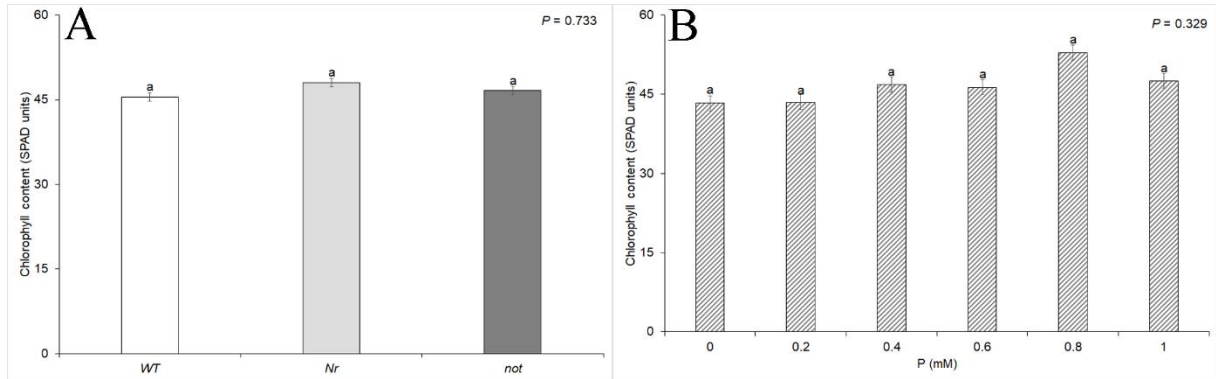


Fig. 2. Chlorophyll content from *S. lycopersicum* genotypes grown under different P concentrations. (A) Comparison between genotypes [*WT* - wild type, *Nr* – Never ripe (lower sensitivity to ethylene) or *not* – *notabilis* (deficient in ABA production)]; (B) effect of P. The Means followed by the same letter do not differ according to the Scott–Knott test for $P < 0.05$. ($n = 72$).

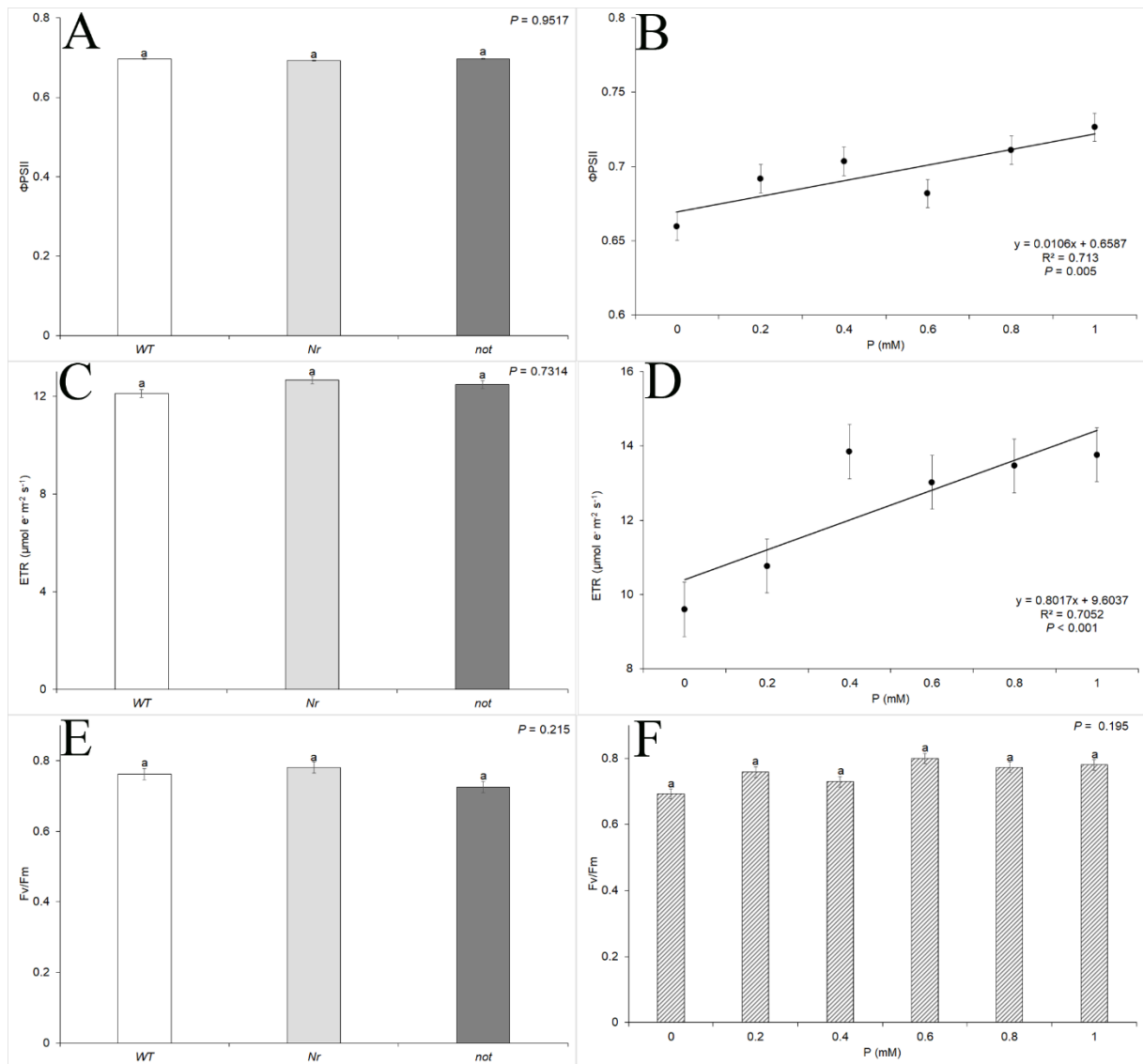


Fig. 3. Photochemical parameters from *S. lycopersicum* genotypes grown under different P concentrations. *WT* - wild type, *Nr* - *Never ripe* (lower sensitivity to ethylene) or *not* - *notabilis* (deficient in ABA production). A and B = quantum yield of the photosystem II, C and D = Electron transfer rate, E and F = maximum yield of the photosystem II. In panels A, C, E and F, means followed by the same letter do not differ according to the Scott-Knott test for $P < 0.05$ and the panel's B and D regression analysis $P < 0.05$. Bars = standard error. ($n = 72$).

The open stomata proportion was highest in the *notabilis* (defective in ABA production) genotype and lowest in the *WT* genotype with *Nr* (lower sensitivity to ethylene) showing intermediate means (Fig. 4A and Fig. 5). The increase in P concentrations increased the open stomata proportion of the *WT* until 0.6 mM but decreased this parameter under 0.8 and 1.0 mM of P (Fig. 4B and Fig. 5A, B). The increase in the P concentration increased the open stomata proportion in the *Nr* genotype showing a linear response (Fig. 4C and Fig. 5C, D). The P

concentrations promoted no significant modification in the open stomata proportion of the *notabilis* genotype (Fig. 4D and 5E, F).

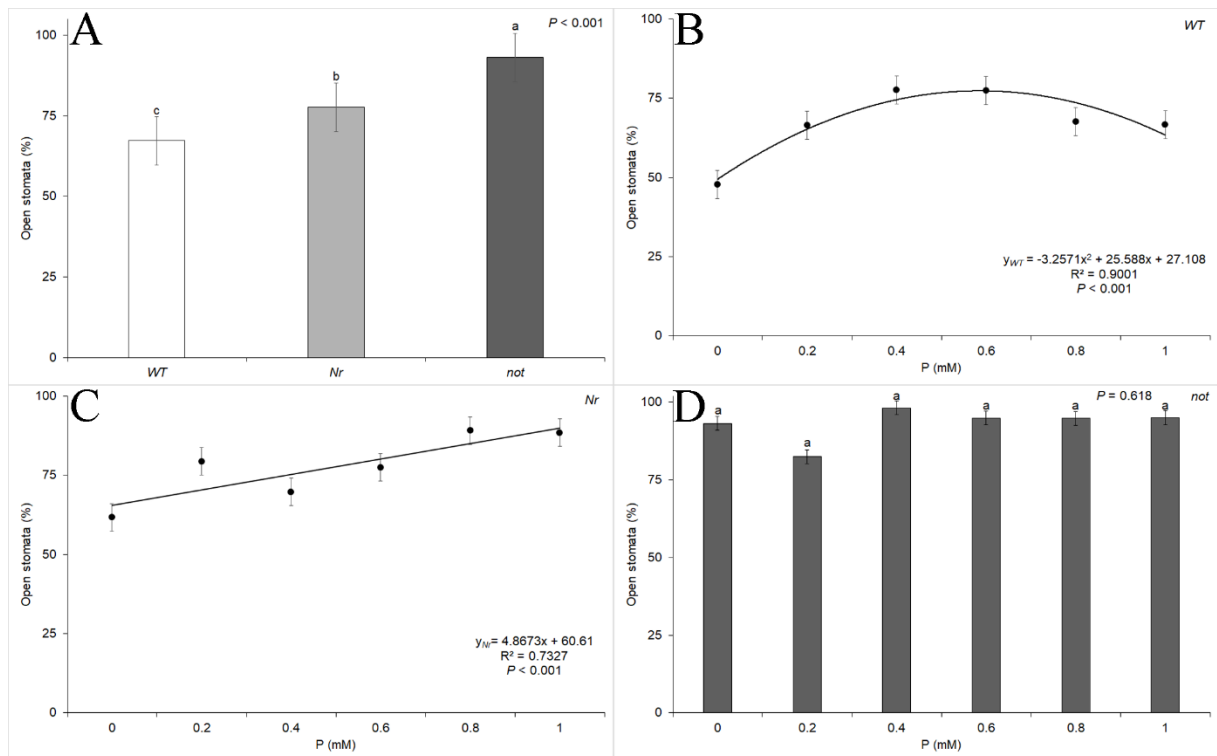


Fig. 4. Open stomata proportion from *S. lycopersicum* genotypes grown under different P concentrations. *WT* - wild type, *Nr* – never ripe (lower sensitivity to ethylene) or *not* – *notabilis* (deficient in ABA production). In panels A and D, means followed by the same letter do not differ according to the Scott–Knott test for $P < 0.05$ and the panels B and C regression analysis $P < 0.05$. Bars = standard error. (n = 72).

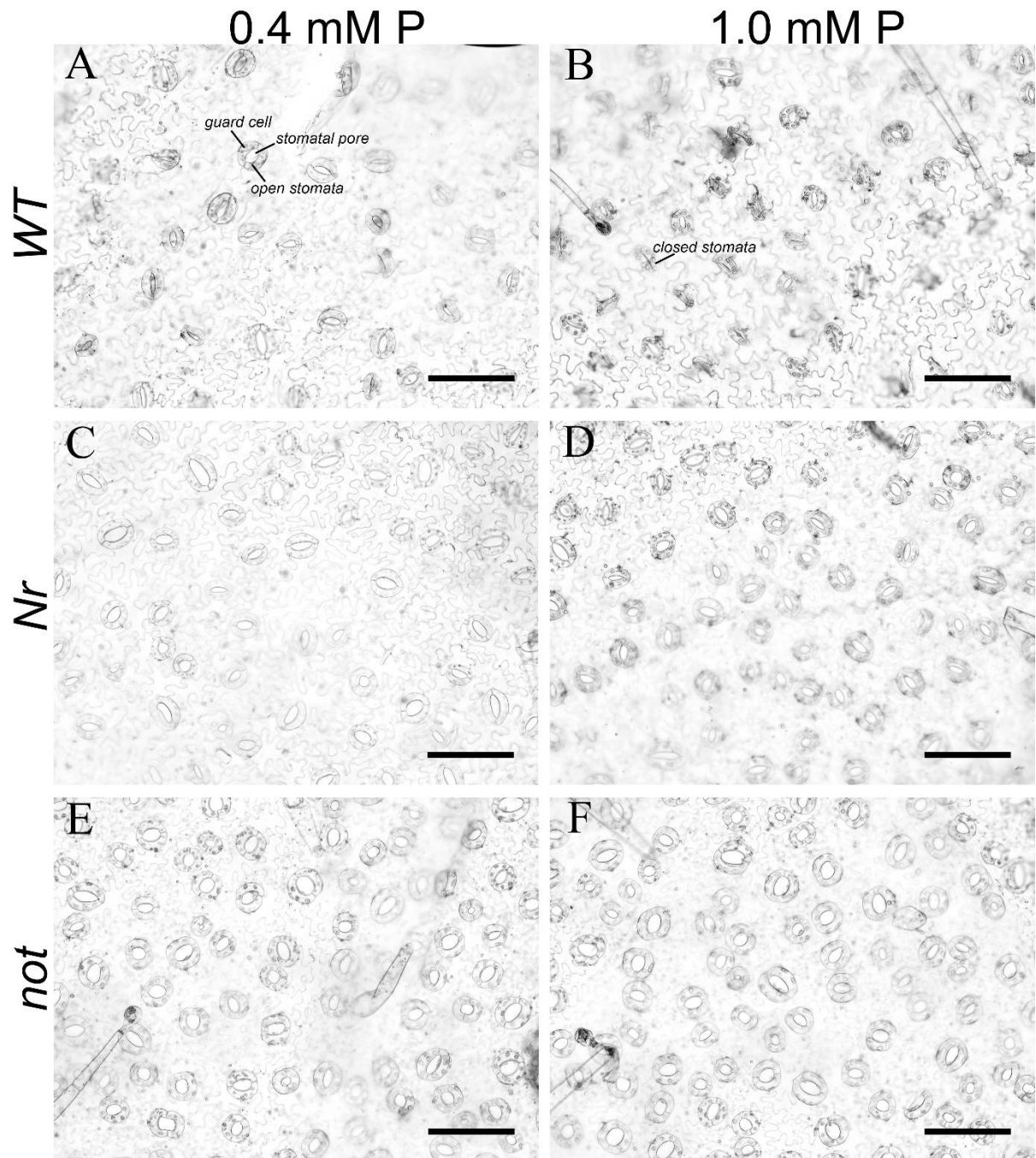


Fig. 5. Abaxial epidermis view from *S. lycopersicum* genotypes grown under different P concentrations. *WT* - wild type, *Nr* – never ripe (lower sensitivity to ethylene) or *not* – *notabilis* (deficient in ABA production). In panels A, C, and E plants grown under 0.4 mM of P (adequate concentration). In panels B, D, and F plants grown under 1.0 mM of P (excessive concentration). Bars = 100 μ m.

The closed stomata proportion was highest in the *WT* genotype and lowest in the *notabilis* genotype, which is deficient in ABA production and showed means 4.7 times smaller than *WT*, the *Nr* (lower sensitivity to ethylene) genotype showed intermediate values (Fig. 6A and Fig. 5). P initially reduced the closed stomata proportion of the *WT* genotype but increased

the closed stomata at 0.6 mM or higher P concentrations (Fig. 6B and Fig. 5A, B). P concentrations decreased the closed stomata proportion in the *Nr* genotype (Fig. 6C and Fig. 5C, D) and promoted no significant modifications in this parameter in the *notabilis* genotype (Fig. 6D and Fig. 5E, F).

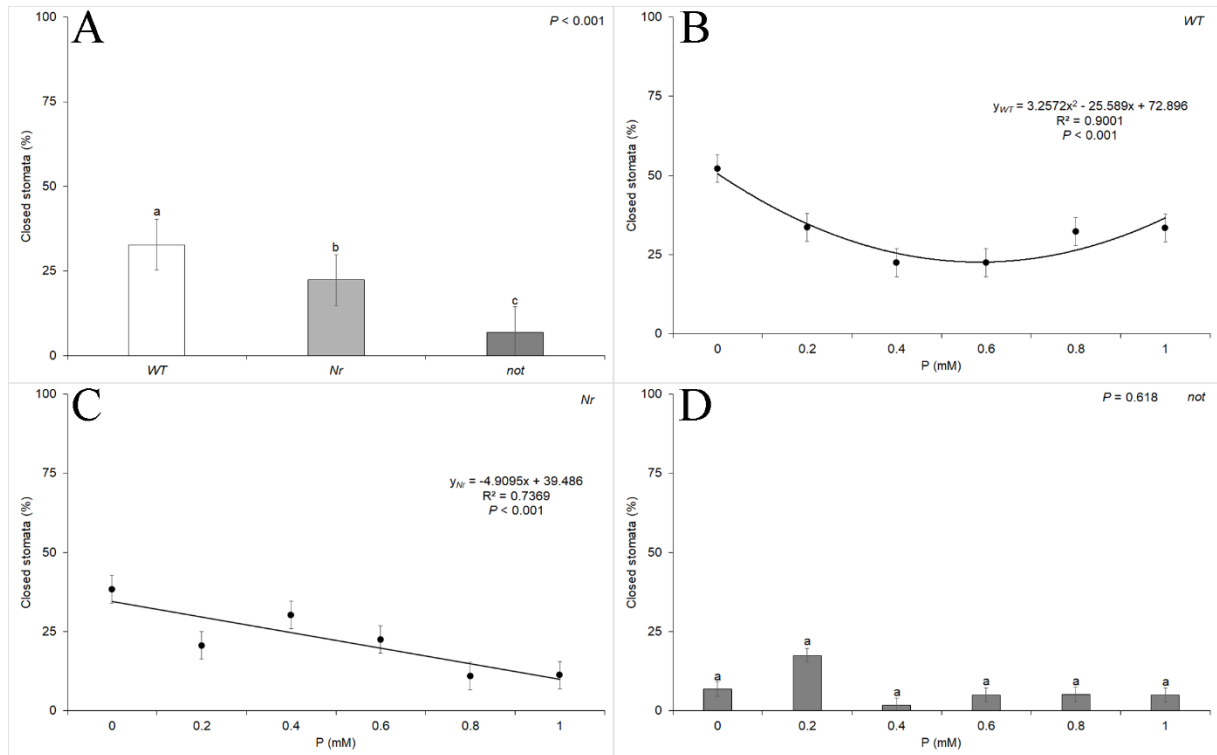


Fig. 6. Closed stomata proportion from *S. lycopersicum* genotypes grown under different P concentrations. *WT* - wild type, *Nr* – *Never ripe* (lower sensitivity to ethylene) or *not* – *notabilis* (deficient in ABA production). A = quantum yield of the photosystem II, B = Electron transfer rate. In panels C and D, means followed by the same letter do not differ according to the Scott–Knott test for $P < 0.05$ and the panels A and B regression analysis $P < 0.05$. Bars = standard error. ($n = 72$).

The stomatal pore area was largest in the *notabilis* genotype (deficient in ABA production) and smallest in the *WT* genotype and the *Nr* (lower sensitivity to ethylene) showed intermediate means (Fig. 7A and Fig. 5). The stomatal pore area from the *notabilis* genotype was 2.4 times larger compared with *WT* and 1.34 times the size of the *Nr* genotype (Fig. 7A and Fig. 5). The stomatal pore area from the *WT* genotype increased with phosphorus concentrations until 0.6 mM but higher concentrations decreased this parameter (Fig. 7B and Fig. 5A, B). Increased P levels enlarged the stomatal pore area in the *Nr* genotype proportionally (Fig. 7C and Fig. 5C, D) but have no effect in this parameter for the *notabilis* genotype (Fig. 7D and Fig. 5E, F).

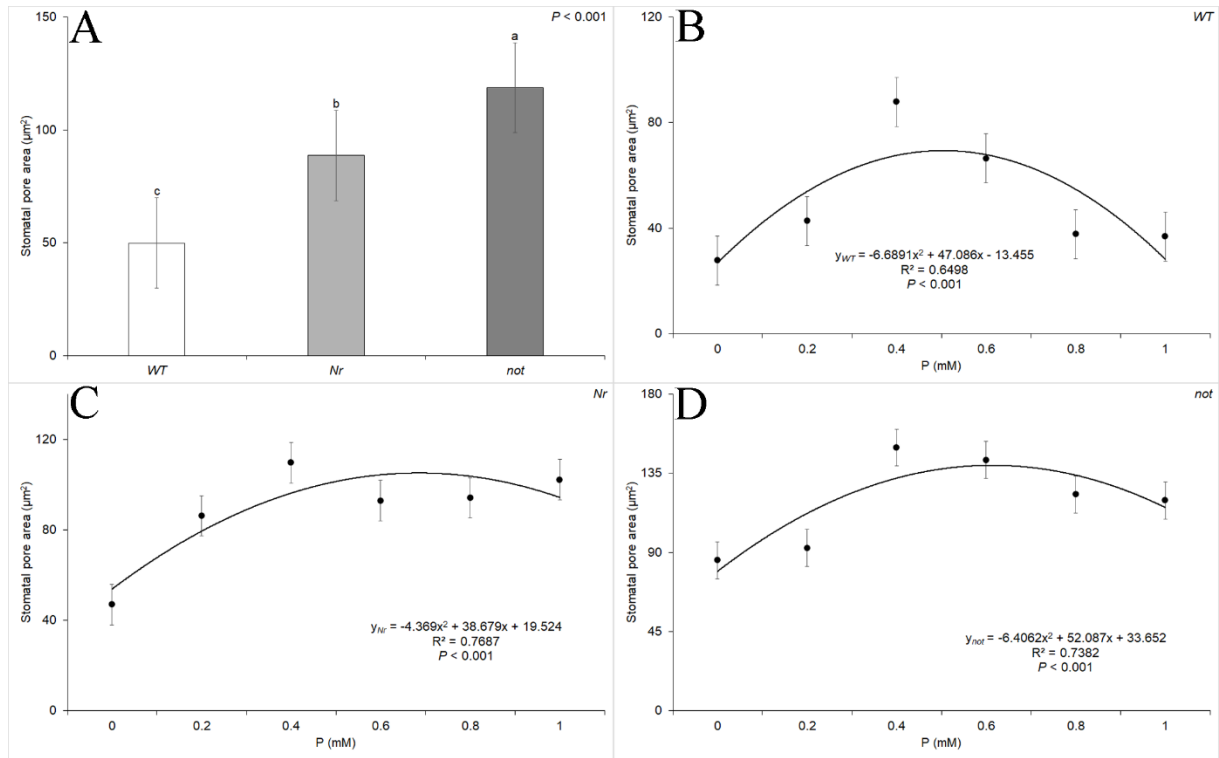


Fig. 7. Stomatal pore area from *S. lycopersicum* genotypes grown under different P concentrations. *WT* - wild type, *Nr* – *Never ripe* (lower sensitivity to ethylene) or *not* – *notabilis* (deficient in ABA production). In panel A means followed by the same letter do not differ according to the Scott–Knott test for $P < 0.05$ and the panels B, C, and D regression analysis $P < 0.05$. Bars = standard error. ($n = 72$).

The *notabilis* genotype showed the largest stomatal density compared to *WT* and *Nr* which showed similar means (Fig. 8A and Fig. 5). P increased the stomatal density of the *WT* genotype until the 0.6 mM concentration but reduced this parameter at higher P levels (Fig. 8B and Fig. 5A, B). P concentrations proportionally increased the stomatal density in the *Nr* genotype (Fig. 8C and Fig. 5C, D). P promoted no significant modification in the stomatal density of *notabilis* genotypes (Fig. 8D and Fig. 5E, F).

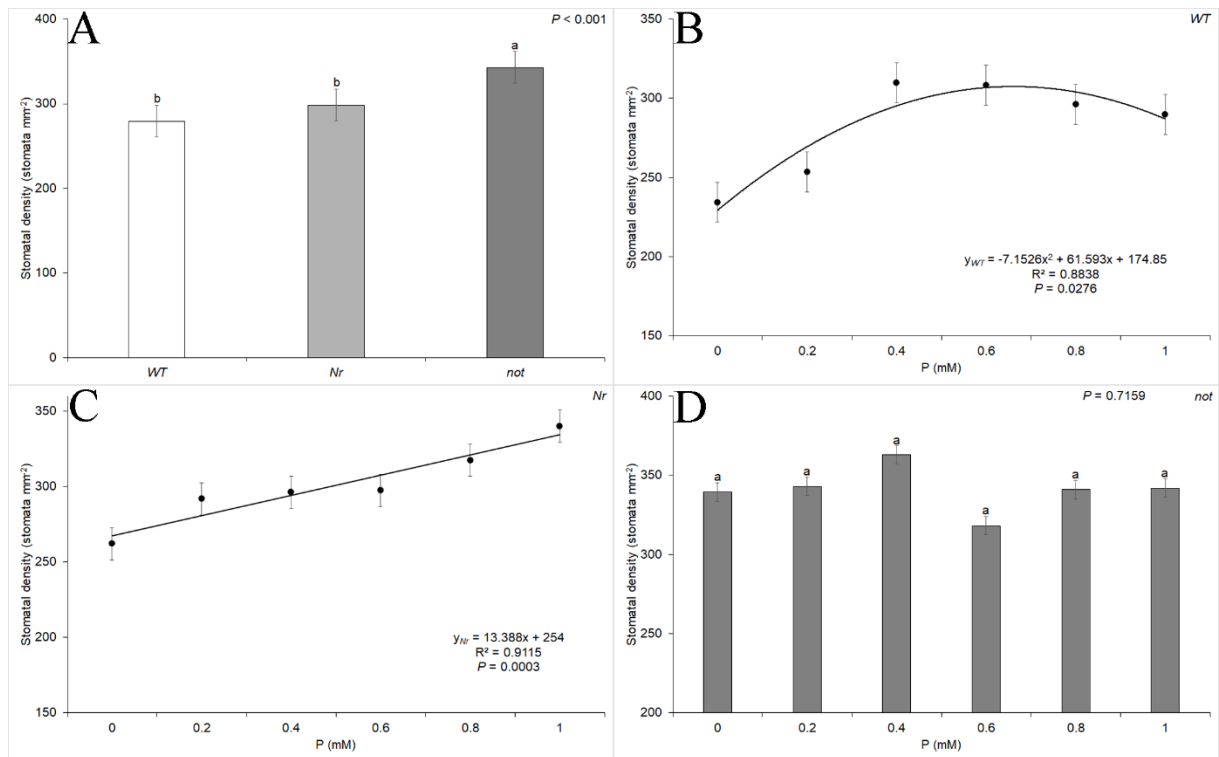


Fig. 8. Stomatal density from *S. lycopersicum* genotypes grown under different P concentrations. *WT* - wild type, *Nr* – *Never ripe* (lower sensitivity to ethylene) or *not* – *notabilis* (deficient in ABA production). In panels A and D the means followed by the same letter do not differ according to the Scott–Knott test for $P < 0.05$ and in panels B and C regression analysis $P < 0.05$. Bars = standard error. ($n = 72$).

The highest stomatal conductance was observed in the *notabilis* genotype (deficient in ABA production) which was 123.9% the values found for the *WT* genotype that showed the lowest stomatal conductance, the *Nr* genotype (lower sensitivity to ethylene) showed intermediate means that were still 62% higher than *WT* means. (Fig. 9A). P increased the stomatal density in the *WT* genotype until 0.6 mM but decreased this parameter at higher levels (Fig. 9B). P proportionally increased the stomatal density from *Nr* (lower sensitivity to ethylene) (Fig. 9C). The element promoted no significant effect in this parameter in the *notabilis* genotype (deficient in ABA production) (Fig. 9D).

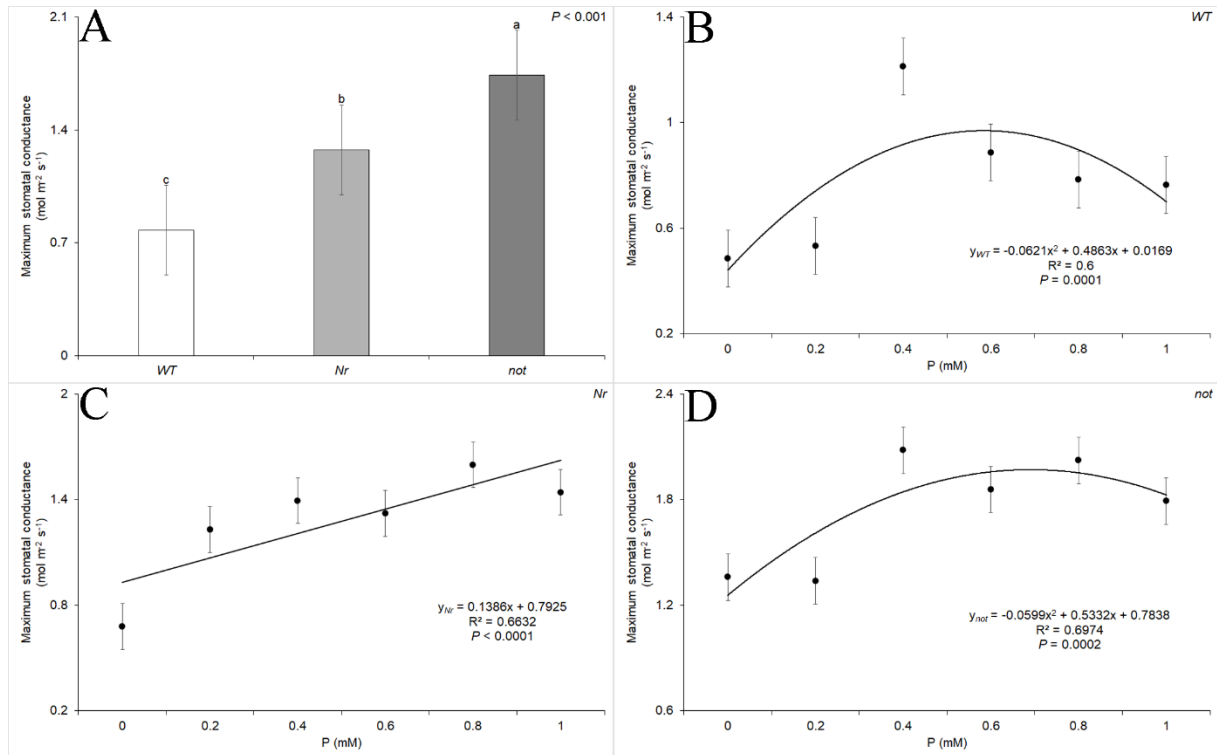


Fig. 9. Stomatal conductance from *S. lycopersicum* genotypes grown under different P concentrations. *WT* - wild type, *Nr* – *Never ripe* (lower sensitivity to ethylene) or *not* – *notabilis* (deficient in ABA production). In panel A means followed by the same letter do not differ according to the Scott–Knott test for $P < 0.05$ and the panels B, C, and D regression analysis $P < 0.05$. Bars = standard error. ($n = 72$).

Transpiration was 29% greater in the *notabilis* genotype (deficient in ABA production) when compared with *WT* and *Nr* genotypes which showed similar means (Fig. 10A). P increased the transpiration of the *WT* genotype until the 0.6 mM but this parameter was reduced at 1 mM of P (Fig. 10B). P increased the transpiration from the *Nr* genotype proportionally to P concentration (Fig. 10C). In the *notabilis* genotype P increased the transpiration until 0.6 mM (Fig. 10D).

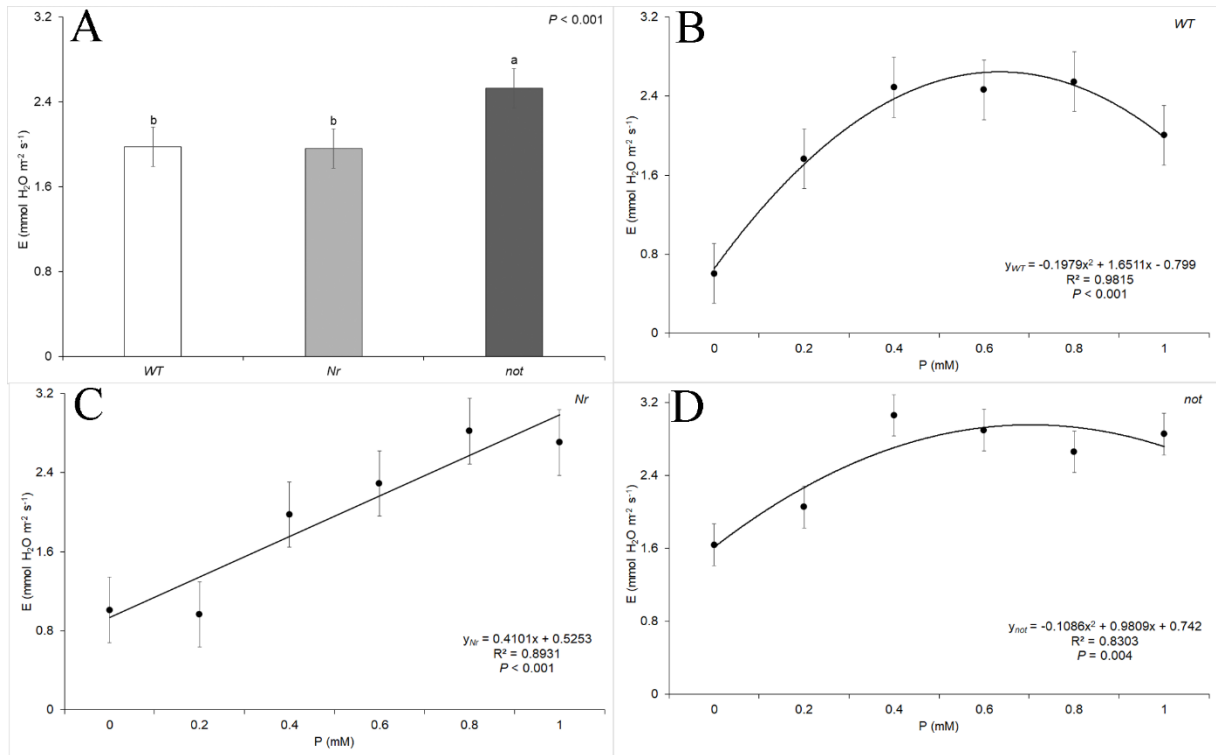


Fig. 10. Leaf transpiration from *S. lycopersicum* genotypes grown under different P concentrations. *WT* - wild type, *Nr* – never ripe (lower sensitivity to ethylene) or *not* – *notabilis* (deficient in ABA production). In panel A means followed by the same letter do not differ according to the Scott–Knott test for $P < 0.05$ and panels B, C, and D regression analysis $P < 0.05$. Bars = standard error. ($n = 72$).

The *Nr* (lower sensitivity to ethylene) showed lower H₂O₂ concentration in leaf tissues. The *WT* and *notabilis* genotypes showed similar means (Fig. 11A). P decreased the H₂O₂ concentration in the *WT* genotype until 0.4 mM but higher concentrations of the nutrient increased the H₂O₂ content (Fig. 11B). The H₂O₂ was not significantly affected by P concentrations in the *Nr* genotype (Fig. 11C). P increased H₂O₂ concentration in leaf tissues of the *notabilis* genotype at 0.4 and 0.6 mM but H₂O₂ decreased at higher P levels (Fig. 11D).

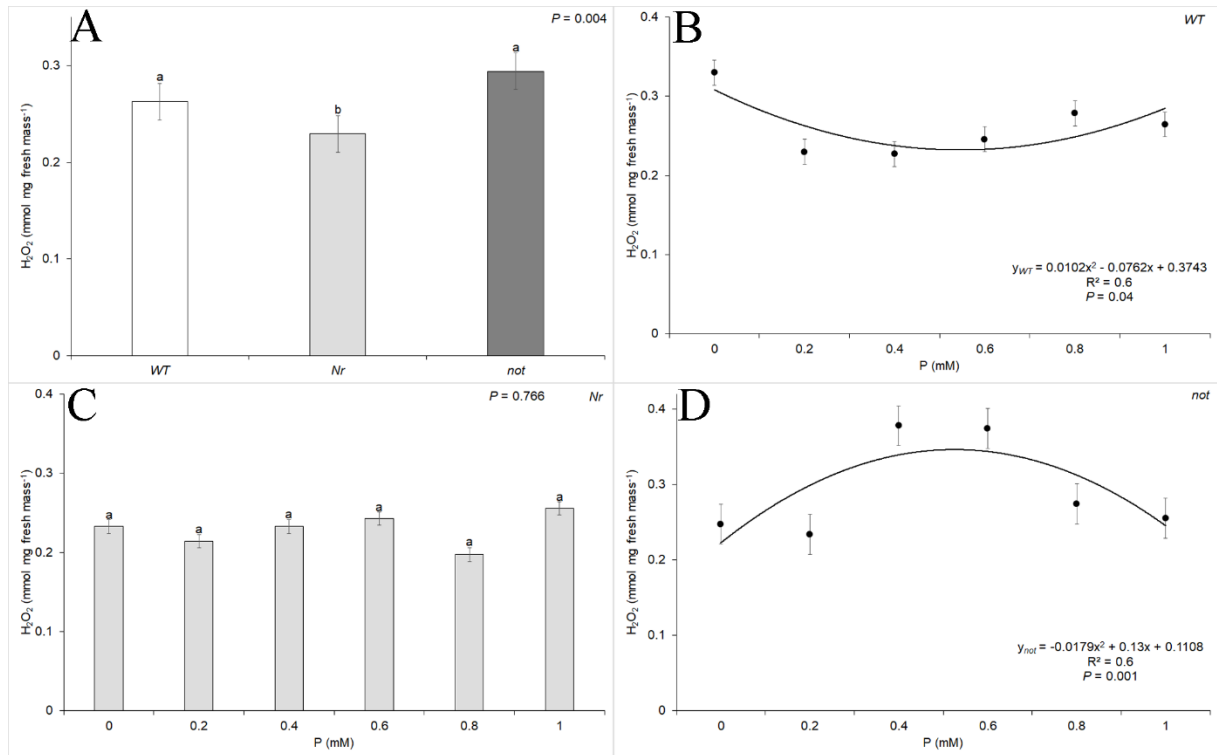


Fig. 11. Leaf H₂O₂ content from *S. lycopersicum* genotypes grown under different P concentrations. *WT* - wild type, *Nr* – never ripe (lower sensitivity to ethylene) or *not* – *notabilis* (deficient in ABA production). In panels A and C the means followed by the same letter do not differ according to the Scott–Knott test for $P < 0.05$ and in panels B and D regression analysis $P < 0.05$. Bars = standard error. ($n = 72$).

4. Discussion

4.1 Excessive P effect in the photosynthesis

Results showed that excessive P promoted negative effects on the dry mass of the *Wt* genotype, suggesting that P levels higher than 0.4 mM disrupt the photosynthetic processes. Photosynthesis is the main process for biomass production in plants and a reduction in the photosynthetic activity will decrease the dry production (Baslam et al., 2020; Lu et al., 2024). Excessive P levels decrease the photosynthesis and growth in different plant species. For instance, concentrations of 0.6 mM or higher of P reduce the photosynthesis and root growth of *Typha domingensis* Pers. grown in nutrient solution (Santos et al., 2015; Santos et al., 2021). Excessive P in soils reduced the growth and development of the tree species *Populus alba* L. (Zakrzewska et al., 2020). P concentrations of 1% or higher in tissues of *Solanum lycocarpum* L. cause toxicity (Jones, 1998). Thus, excessive P concentrations decrease the dry mass of *S. lycopersicum* due to a photosynthetic limitation.

Despite the reduction of the dry mass of the *WT* genotype under high P concentrations, the *notabilis* genotype (which is deficient in ABA production) was not affected by high P levels and the *Nr* genotype (with lower sensitivity to ethylene) showed no growth reduction and even increased its dry mass under 0.8 mM. These results suggest that the photosynthesis of the *Nr* genotype was less affected by excessive P and the absence of negative responses in the *notabilis* genotype, which is deficient in ABA production suggests that the photosynthetic limitation is related to the stomatal closing. The lack of negative effects from excessive P concentrations in the *Nr* genotype suggests that this phytohormone is associated with negative responses in dry mass production. Ethylene is likely signaling excessive P in roots leading to an increase in ABA synthesis that can be transported to leaves and cause stomatal closure, limiting CO₂ acquisition and photosynthesis. According to Shukla et al. (2017), excessive P increases ethylene production and signaling in *Arabidopsis thaliana* L. In addition, Santos et al. (2015) showed reduced stomatal conductance related to lower photosynthesis under excessive P concentrations (above 0.6 mM) in *T. domingensis*. Results suggest that P concentrations higher than 0.6 mM stimulate ethylene synthesis which leads to ABA production in roots and stomatal closure, limiting the biochemical stage of photosynthesis in *S. lycopersicum*.

The results for the photochemical parameters of *S. lycopersicum* genotypes support our hypothesis that the negative effects in photosynthesis caused by excessive P levels are related to the biochemical stage. The photochemical results exhibited no negative responses by P and still the dry mass production was reduced, this suggests that the limitation to photosynthesis is related to the Calvin-Benson cycle and is likely to reduce CO₂ acquisition by the stomata. The photochemical stage of photosynthesis produces ATP and NADPH which are used further in the Calvin-Benson cycle. The chlorophyll content is a key feature of the photochemical stage and this parameter and the Fv/Fm were not significantly modified by P concentrations in *S. lycopersicum* genotypes. This suggests that excessive P did not trouble the photochemical stage of *S. lycopersicum*. The reduction in the chlorophyll content is directly related to photosynthesis decrease for several species and environmental factors, such as for *T. domingensis* under water limitation (Cruz et al., 2019) and *Schinus molle* L. submitted to Cd stress (Pereira et al., 2016). In addition, the reduction of the Fv/Fm is also related to lower photosynthesis as shown by *Cyclocarya paliurus* (Batalin) Iljinsk. (Liu et al., 2018). These results suggest that the lower biomass production and photosynthesis under excessive P levels observed in the *Wt* genotype can not be explained by the photochemical traits and must be related to CO₂ limitation possibly caused by stomatal closure that decreased the carboxylation in the Calvin-Benson cycle.

Results showed that the ϕ PSII and ETR increased in all *S. lycopersicum* genotypes, corroborating the hypothesis that the photochemical stage can not be related to the reduced dry mass in the *Wt* genotype. The increase in the ϕ PSII and ETR is often associated with higher photosynthetic rates as shown by *C. paliurus* (Liu et al., 2018). In addition, reducing these parameters decreases the photosynthesis and dry mass production in several species including *S. lycopersicum* (Lu et al., 2017; Tian et al., 2017). Therefore, the reduced dry mass production and photosynthesis observed in the *Nr* genotype must be related to limited CO₂ acquisition by stomata and a limitation to the Calvin-Benson cycle.

4.2 Excessive P effect in the stomatal closure

P concentrations promoted specific stomatal responses for each *S. lycopersicum* genotype. The *notabilis* genotype showed a lower percentage of closed stomata compared with *WT* and *Nr*, which may related to the mutation that lowers ABA production and accumulation in *notabilis* (Burbidge et al., 1999). ABA is a fundamental phytohormone for regulating stomatal closure under environmental stress (Bharath et al., 2021; Liu et al., 2022; Haworth et al., 2018). ABA deficiency can also explain the higher transpiration and stomatal conductance in *notabilis* compared to *Nr* and *WT* (Fig. 9 and Fig. 10). The higher stomatal density in *notabilis* compared to *Nr* and *WT* can also be related to lower ABA levels because it can limit stomatal development. ABA-deficient mutants in different plant species often show higher stomatal density (Tanaka et al., 2013; Merilo et al., 2018). Stomatal density limits water loss using a different mechanism compared to ABA-induced stomatal closure related to *OST1* receptors in guard cells (Jalakas et al., 2018). Thus, the regulation of stomatal density in *S. lycopersicum* depends on ABA production.

P concentrations of 0.6 mM or higher reduced the stomatal density in *WT* whereas this effect was not evident in *notabilis* (deficient in ABA production) and *Nr* (lower sensitivity to ethylene) (Fig. 8). These results indicate that excessive P lowers the stomatal density depending on ABA and ethylene. Lower ABA production in plants reduces the stomatal density (Tanaka et al., 2013; Jalakas et al., 2018; Merilo et al., 2018) and the absence of significant modifications in stomatal density from *notabilis* mutant suggests that excessive P increases ABA production in *WT* reducing its stomatal density. Because there was no reduction of the stomatal density in *Nr* it also indicates that the regulation of stomatal density is related to ethylene. Ethylene production stimulates the synthesis of ABA (Grossmann and Hansen, 2000) and excessive P

concentrations increase ethylene production (Shukla et al., 2017). Thus, ethylene production can be stimulated at excessive P levels which increase ABA synthesis, reducing the stomatal density.

This study hypothesizes that excessive P levels stimulate the ethylene synthesis increasing ABA production and causing stomatal closure, reducing photosynthesis and growth. Ethylene directly regulates stomatal closure by increasing cytoplasmic levels of reactive oxygen species (ROS) mainly H_2O_2 which reduces cytoplasmic K^+ and increases Ca^{2+} (Desikan et al., 2006; Liu et al., 2010). Ethylene also stimulates ABA production (Grossmann and Hansen, 2001) and ABA also regulates stomatal closure using H_2O_2 signals to reduce cytoplasmic K^+ and increase Ca^{2+} (An et al., 2016). Increased H_2O_2 in leaves was observed in *WT* under 0.6 mM or higher P levels but this effect was not observed in either *notabilis* (deficient in ABA production) and *Nr* (lower sensitivity to ethylene) mutants (Fig. 11). Thus, results indicate that stomatal closure in *S. lycopersicum* under excessive P concentrations is related to ethylene production which stimulates ABA synthesis and both phytohormones may promote H_2O_2 formation which is related to signaling for stomatal closure.

The reduction of the stomatal pore area and the higher percentage of closed stomata only in the *WT* genotype under excessive P levels support the hypothesis that stomatal closure under these conditions is related to ethylene and ABA production. The stomatal closure promoted by high P levels is also supported by lower stomatal conductance (Fig. 9) and transpiration (Fig. 10). Stomatal closure and the reduction of its pore area are related to lower stomatal conductance in different plant species (Kaiser, 2009; Poór et al., 2019). Santos et al. (2015) showed that excessive P levels reduced the stomatal conductance, transpiration, and photosynthesis in *T. domingensis*. Thus, the absence of reduction in the stomatal conductance and transpiration in the *Nr* (lower sensitivity to ethylene) and *notabilis* (deficient in ABA production) also supports the hypothesis that stomatal closure under excessive P levels is related to ethylene and ABA.

4.3 Excessive P signaling to guard cells

Results showed that excessive P promoted stomatal closure in the *WT* genotype. However, this effect was negligible for either *Nr* (lower sensitivity to ethylene) or *notabilis* (which is deficient in ABA production) mutants. In addition, excessive P increased H_2O_2 concentration in leaves from *WT* only. These results suggest that ethylene and ABA are related

to stomatal closure by H₂O₂ signaling. Ethylene promotes stomatal closure using H₂O₂ as a signaling molecule in membrane receptors from cell guards (Desikan et al., 2006; Liu et al., 2010). In addition, excessive P increases the ethylene synthesis (Shukla et al., 2017). The absence of significant stomatal closure under higher P levels in the *Nr* mutant supports the hypothesis that ethylene signaling is related to stomatal closure. This genotype is defective in the ethylene receptor *LeETR3* becoming less sensitive to this phytohormone (Carvalho et al., 2011). This suggests a direct ethylene-dependent signaling pathway promoted by excessive P causing stomatal closing (Fig. 12). The absence of significant stomatal closing and H₂O₂ production under excessive P in *notabilis* genotype also suggests an alternative signaling route because this genotype is defective in the carotenoid-cleaving enzyme 9-cis-epoxy carotenoid dioxygenase and therefore decreases the production and accumulation ABA (Carvalho et al., 2011). This suggests an ABA-dependent mechanism that may be triggered by ethylene production under higher P levels (Fig. 12). ABA signaling for stomatal closure can also use H₂O₂ as a signaling molecule (An et al., 2016), and ABA synthesis is stimulated by ethylene (Grossmann and Hansen, 2001). Thus, results suggest excessive P promotes stomatal closure by either ethylene-dependent or ABA-dependent pathways mediated by H₂O₂ in cell guards.

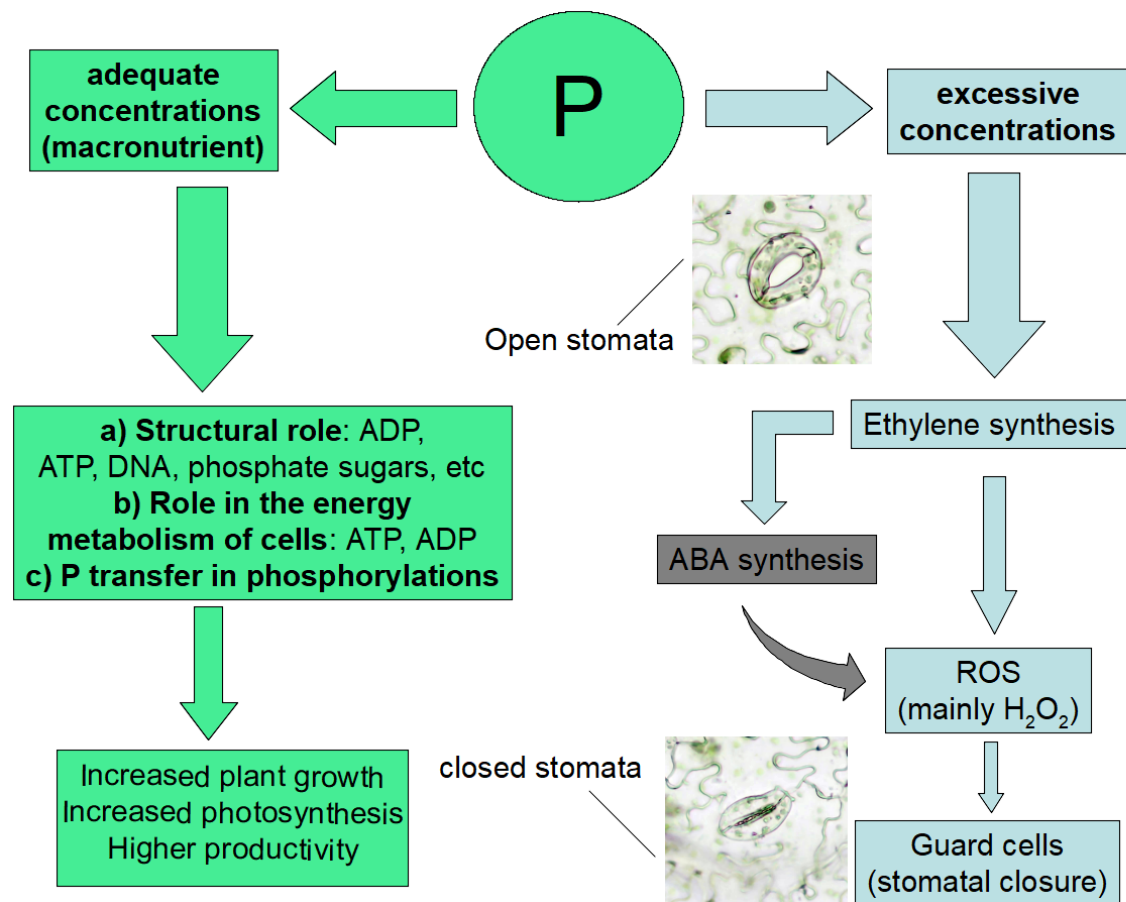


Fig. 12. Ethylene and ABA-dependent pathways for stomatal closure under excessive P concentrations.

Conclusion

Excessive P promotes stomatal closure limiting photosynthesis by a reduction in the CO₂ uptake. Excessive P concentrations reduce stomatal conductance and transpiration but promote no significant changes in the photochemical parameters in *S. lycopersicum*. Excessive P concentrations increase the percentage of closed stomata and reduce the stomatal pore area in *S. lycopersicum*. Excessive P concentrations increase the H₂O₂ content in the leaves of *S. lycopersicum*. The absence of effects of excessive P levels in the *Nr* (lower sensitivity to ethylene) and *notabilis* (deficient in ABA production) indicates that stomatal closure related to excessive P depends on ethylene and ABA signaling to guard cells.

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References

Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant Cell Environ* 24: 1337-1344. <https://doi.org/10.1046/j.1365-3040.2001.00778.x>

An Y, Liu L, Chen L, Wang L (2016) ALA inhibits ABA-induced stomatal closure via reducing H₂O₂ and Ca²⁺ levels in guard cells. *Frontiers Plant Sci* 7: 1-16. <https://doi.org/10.3389/fpls.2016.00482>

Assuero SG, Mollier A, Pellerin S (2004) The decrease in growth of phosphorus-deficient maize leaves is related to a lower cell production. *Plant Cell Environ* 27: 887-895. <https://doi.org/10.1111/j.1365-3040.2004.01194.x>

Baslam M, Mitsui T, Hodges M, Priesack E, Herritt MT, Aranjuelo I, Sanz-Sáez Á (2020) Photosynthesis in a changing global climate: scaling up and scaling down in crops. *Frontiers Plant Sci* 11: 1-29. <https://doi.org/10.3389/fpls.2020.00882>

Burbidge A, Grieve TM, Jackson A, Thompson A, McCarty DR, Taylor IB (1999) Characterization of the ABA deficient tomato mutant notabilis and its relationship with maize Vp14. *Plant J* 17: 427-431. <https://doi.org/10.1046/j.1365-313x.1999.00386.x>

Bharath P, Gahir S, Raghavendra A (2021) Absciscic acid-induced stomatal closure: an important component of plant defense against abiotic and biotic stress. *Frontiers Plant Sci* 12: 1-18. <https://doi.org/10.3389/fpls.2021.615114>

Carvalho RF, Campos ML, Pino LE, Lombardi-Crestana SL, Zsogon A, Lima JE, Benedito VA, Peres LEP (2011) Convergence of developmental mutants into a single tomato model system: Micro-Tom as an effective toolkit for plant development research. *Plant Methods* 7: 1-14. <https://doi.org/10.1186/1746-4811-7-18>

Chiera J, Thomas J, Rufty T (2002) Leaf initiation and development in soybean under phosphorus stress. *J Exp Bot* 53: 473-481. <https://doi.org/10.1093/jexbot/53.368.473>

Corrêa FF, Pereira MP, Kloss RB, Castro EM, Pereira FJ (2017) Leaf ontogeny and meristem activity of *Typha domingensis* Pers. (Typhaceae) under different phosphate concentrations. *Aquat Bot* 136: 43-51. <http://dx.doi.org/10.1016/j.aquabot.2016.09.007>

Cruz YC, Scarpa ALM, Pereira MP, Castro EM, Pereira FJ (2019) Growth of *Typha domingensis* as related to leaf physiological and anatomical modifications under drought conditions. *Acta Physiol Plant* 41: 1-9. <https://doi.org/10.1007/s11738-019-2858-1>

Desikan R, Last K, Harrett-Williams R, Tagliavia C, Harter K, Hooley R, Hancock JT, Neill SJ (2006) Ethylene-induced stomatal closure in Arabidopsis occurs via AtrbohF-mediated hydrogen peroxide synthesis. *Plant J* 47: 907-916. <https://doi.org/10.1111/j.1365-313x.2006.02842.x>

Duarte VP, Pereira MP, Correa F, Castro EM, Pereira FJ (2021) Aerenchyma, gas diffusion, and catalase activity in *Typha domingensis*: a complementary model for radial oxygen loss. *Protoplasma* 258: 765-777. <https://doi.org/10.1007/s00709-020-01597-8>

Ferreira DF (2011) Sisvar: a computer statistical analysis system. *Cien Agrotecnol* 35: 1039-1042. <http://dx.doi.org/10.1590/S1413-70542011000600001>

Franks PJ, Beerling DJ (2009) Maximum leaf conductance driven by CO₂ effects on stomatal size and density over geologic time. *PNAS* 106: 10343-10347. <https://doi.org/10.1073/pnas.0904209106>

Grossmann K, Hansen H (2001) Ethylene-triggered abscisic acid: a principle in plant growth regulation? *Physiol Plant* 113: 9-14. <http://dx.doi.org/10.1034/j.1399-3054.2001.1130102.x>

Gruber BD, Giehl RFH, Friedel S, Von Wirén N (2013) Plasticity of the *Arabidopsis* root system under nutrient deficiencies. *Plant Physiol* 163: 161-179. <https://doi.org/10.1104/pp.113.218453>

Gay AP, Hurd RG (1975) The influence of light on stomatal density in the tomato. *New Phytol* 75: 37-46. <https://doi.org/10.1111/j.1469-8137.1975.tb01368.x>

Haworth M, Marino G, Cosentino SL, Brunetti C, De Carlo A, Avola G, Riggi E, Loreto F, Centritto M (2018) Increased free abscisic acid during drought enhances stomatal sensitivity and modifies stomatal behaviour in fast growing giant reed (*Arundo donax* L.). *Environ Exp Bot* 147: 116-124. <https://doi.org/10.1016/j.envexpbot.2017.11.002>

Hackett RM, Ho CW, Lin ZF, Foote HCC, Fray RG, Grierson D (2000) Antisense inhibition of the *Nr* gene restores normal ripening to the tomato Never-ripe mutant, consistent with the ethylene receptor-inhibition model. *Plant Physiol* 124: 1079-1086. <https://doi.org/10.1104%2Fpp.124.3.1079>

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. *Calif Agric Exp Stn* 347: 1-39.

Jalakas P, Merilo E, Kollist H, Brosché M (2018) ABA-mediated regulation of stomatal density is OST1-independent. *Plant Direct* 2: 1-7. <https://doi.org/10.1002/pld3.82>

Jones JB (1998) Phosphorus toxicity in tomato plants: when and how does it occur? *Comm Soil Sci Plant Anal* 29: 1779-1784. <https://doi.org/10.1080/00103629809370068>

Kaiser H (2009) The relation between stomatal aperture and gas exchange under consideration of pore geometry and diffusional resistance in the mesophyll. *Plant Cell Environ* 32: 1091-1098. <https://doi.org/10.1111/j.1365-3040.2009.01990.x>

Kayoumu M, Iqbal A, Muhammad N, Li X, Li L, Wang X, Gui H, Qi Q, Ruan S, Guo R, Zhang X, Song M, Dong Q (2023) Phosphorus Availability Affects the Photosynthesis and Antioxidant System of Contrasting Low-P-Tolerant Cotton Genotypes. *Antioxidants* 12: 1-23. <https://doi.org/10.3390/antiox12020466>

Liu H, Song S, Zhang H, Li Y, Niu L, Zhang J, Wang W (2022) Signaling transduction of ABA, ROS, and Ca²⁺ in plant stomatal closure in response to drought. *Int J Mol Sci* 23: 1-13. <https://doi.org/10.3390/ijms232314824>

Liu J, Liu G, Hou L, Liu X (2010) Ethylene-induced nitric oxide production and stomatal closure in *Arabidopsis thaliana* depending on changes in cytosolic pH. *Chin Sci Bull* 55: 2403-2409. <http://dx.doi.org/10.1007/s11434-010-4033-3>.

Liu Y, Wang T, Fang S, Zhou M, Qin J (2018) Responses of morphology, gas exchange, photochemical activity of photosystem II, and antioxidant balance in *Cyclocarya paliurus* to light spectra. *Front Plant Sci* 9: 1-15. <http://dx.doi.org/10.3389/fpls.2018.01704>

Lu A, Zeng S, Pi K, Long B, Mo Z, Liu R (2024) Transcriptome analysis reveals the key role of overdominant expression of photosynthetic and respiration-related genes in the formation of tobacco (*Nicotiana tabacum* L.) biomass heterosis. *BMC Gen* 25: 1-14. <https://doi.org/10.1186/s12864-024-10507-8>

Lu T, Meng Z, Zhang G, Qi M, Sun Z, Liu Y, Li T (2017) Sub-high temperature and high light intensity induced irreversible inhibition on photosynthesis system of tomato plant (*Solanum lycopersicum* L.). *Front Plant Sci* 8: 1-16. <https://doi.org/10.3389/fpls.2017.00365>

Meng X, Chen W-W, Wang Y-Y, Huang Z-R, Ye X, Chen L-S, Yang L-T (2021) Effects of phosphorus deficiency on the absorption of mineral nutrients, photosynthetic system performance and antioxidant metabolism in *Citrus grandis*. *Plos One* 16: e0246944. <https://doi.org/10.1371/journal.pone.0246944>

Merilo E, Yarmolinsky D, Jalakas P, Parik H, Tulva I, Rasulov B, Kilk K, Kollist H (2018) Stomatal VPD response: there is more to the story than ABA. *Plant Physiol* 176: 851-864. <https://doi.org/10.1104/pp.17.00912>

Parlange JY, Waggoner PE (1970) Stomatal dimensions and resistance to diffusion. *Plant Physiol* 46: 337-342. <https://doi.org/10.1104/pp.46.2.337>

Pereira FJ, Castro EM, Oliveira C, Pires MF, Pereira MP, Ramos SJ, Faquin F (2014) Lead tolerance of water hyacinth (*Eichhornia crassipes* mart. - Pontederiaceae) as defined by anatomical and physiological traits. *An Acad Bras Cien* 86: 1423-1433. <https://doi.org/10.1590/0001-3765201420140079>

Pereira FJ, Castro EM, Pires MF, Oliveira C, Pasqual M (2017) Anatomical and physiological modifications in water hyacinth under cadmium contamination. *J Appl Bot Food Qual* 90: 10-17 <https://doi.org/10.5073/jabfq.2017.090.003>

Pereira, MP, Rodrigues LCA, Correa F, Castro EM, Ribeiro VE, Pereira FJ (2016) Cadmium tolerance in *Schinus molle* trees is modulated by enhanced leaf anatomy and photosynthesis. *Trees* 30: 807-814. <https://doi.org/10.1007/s00468-015-1322-0>

Poór P, Borbély P, Czékus Z, Takács Z, Ördög A, Popović B, Tari I (2019) Comparison of changes in water status and photosynthetic parameters in wild type and abscisic acid-deficient *siliens* mutant of tomato (*Solanum lycopersicum* cv. Rheinlands Ruhm) exposed to sublethal and lethal salt stress. *J Plant Physiol* 232: 130-140. <https://doi.org/10.1016/j.jplph.2018.11.015>

Santos JCB, Castro EM, Duarte VP, Pereira MP, Correa F, Pereira FJ (2021) Root growth and anatomy of *Typha domingensis* Pers. related to phosphorus availability. *Acta Limnol Bras* 33: e19. <https://doi.org/10.1590/s2179-975x2120>

Santos KR, Pereira MP, Ferreira ACG, Rodrigues LCA, Castro EM, Corrêa FF, Pereira FJ (2015) *Typha domingensis* Pers. responses to leaf anatomy and photosynthesis as influenced by phosphorus. *Aquatic Bot* 122: 47-53. <http://dx.doi.org/10.1016/j.aquabot.2015.01.007>

Sharpley NA, Tunney H (2000) Phosphorus research strategies to meet agricultural and environmental challenges of the 21st century. *J Environ Qual* 29: 176-181. <https://doi.org/10.2134/jeq2000.00472425002900010022x>

Shukla D, Rinehart CA, Sahi SV (2017) Comprehensive study of excess phosphate response reveals ethylene mediated signaling that negatively regulates plant growth and development. *Scientific Reports* 7: 1-16. <https://doi.org/10.1038/s41598-017-03061-9>

Smith VH, Schindler DW (2009) Eutrophication science: where do we go from here? *Trends Ecol Evo* 24: 201-207. <https://doi.org/10.1016/j.tree.2008.11.009>

Tanaka Y, Nose T, Jikumaru Y, Kamiya Y (2013) ABA inhibits entry into stomatal-lineage development in *Arabidopsis* leaves. *Plant J* 74: 448-457. <https://doi.org/10.1111/tpj.12136>

Tian Y, Ungerer P, Zhang H, Ruban A (2017) Direct impact of the sustained decline in the photosystem II efficiency upon plant productivity at different developmental stages. *J Plant Physiol* 212: 45-53. <https://doi.org/10.1016/j.jplph.2016.10.017>

Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants. *Plant Sci* 151: 59-66. [https://doi.org/10.1016/s0168-9452\(99\)00197-1](https://doi.org/10.1016/s0168-9452(99)00197-1)

Xue Y-B, Xiao B-X, Zhu S-N, Mo X-H, Liang C-Y, Tian J, Liao H (2017) *GmPHR25*, a *GmPHR* member up-regulated by phosphate starvation, controls phosphate homeostasis in soybean. *J Exp Bot* 68: 4951-4967. <https://doi.org/10.1093/jxb/erx292>

Zakrzewska J, Mitrović AL, Mutavdžić D, Dučić T, Radotić K (2020) Phosphorus homeostasis in *Populus alba* L. under excess phosphate conditions, assessed by ³¹P nuclear magnetic resonance spectroscopy and X-ray microfluorescence. *Environ Sci Pollut Res* 27: 3320-3328. <https://doi.org/10.1007/s11356-019-07200-9>

Zhang X, Zhang L, Dong F, Gao J, Galbraith DW, Song C-P (2001) Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiol* 126: 1438-1448. <https://doi.org/10.1104/pp.126.4.1438>

Zhang Y-Q, Deng Y, Chen R-Y, Cui Z-L, Chen X-P, Yost R, Zhang F-S, Zou C-Q (2012) The reduction in zinc concentration of wheat grain upon increased phosphorus-fertilization and its mitigation by foliar zinc application. *Plant Soil* 361: 143-152. <https://doi.org/10.1007/s11104-012-1238-z>

TERCEIRA PARTE

1 CONSIDERAÇÕES FINAIS

Essa pesquisa demonstra que a aplicação de fósforo (P) exógeno diretamente na epiderme foliar de *Solanum lycopersicum* desencadeia o fechamento estomático. Os resultados corroboram a hipótese do trabalho, indicando que o fechamento estomático observado é induzido pelo fósforo e mediado pelo etileno. Quando as plantas crescem em substrato com excesso de P, há uma limitação na formação de biomassa. Concentrações excessivas de P aumentam a porcentagem de estômatos fechados, reduzem a área do poro estomático e elevam o conteúdo de H₂O₂ nas folhas de *S. lycopersicum*. Como altas concentrações de P não promovem mudanças significativas nos parâmetros fotoquímicos das plantas, a redução do crescimento em plantas cultivadas em altas concentrações de P provavelmente deve ser causada pela menor captação de CO₂ devido ao fechamento dos estômatos. A menor resposta aos efeitos negativos nos parâmetros estomáticos e de crescimento nos genótipos *Nr* (menor sensibilidade ao etileno) e *notabilis* (deficiência na produção de ABA) sugere que o fechamento estomático promovido pelo excesso de P é desencadeado pela sinalização de etileno e ABA para células guarda. Entender-se, portanto, o limite de P para as plantas pode auxiliar na escolha de limites de aplicação do nutriente para que o impacto da agricultura na eutrofização seja reduzido, além de otimizar a eficiência do uso de fertilizantes fosfatados e reduzir os custos de produção.