INSTITUTO AGRONÔMICO PROGRAMA DE PÓS-GRADUAÇÃO AGRICULTURA TROPICAL E SUBTROPICAL

DESVENDANDO O PAPEL DO ÓXIDO NÍTRICO NA TOLERÂNCIA À SECA EM PLANTAS DE CITROS

STEPHANIE ESTETE PEREIRA

Orientadora: Dra. Neidiquele Maria Silveira

Dissertação apresentada como requisito para a obtenção do título de **Mestre** em Agricultura Tropical e Subtropical, Área de Concentração em Sistemas de Manejo e Qualidade (SMQA).

Campinas, SP
2026

Ficha catalográfica elaborada pela bibliotecária da Seção Documentação Científica do Instituto Agronômico

P436d Pereira, Stephanie Estete
Desvendando o papel do óxido nítrico na tolerância à seca em plantas de citros. /Stephanie Estete Pereira. Campinas, 2026.
32 fls

Orientadora: Neidiquele Maria Silveira
Dissertação (Mestrado) em Agricultura Tropical e Subtropical-Instituto Agronômico

1. Transporte de água. 2. Stresse oxidativo. 3. PIP1. 4. PIP2. 4. Déficit hídrico. I.Silveira, Neidiquele Maria. I. Título.

CDD. 634.3

GOVERNO DO ESTADO DE SÃO PAULO
SECRETARIA DE AGRICULTURA E ABASTECIMENTO
AGÊNCIA PAULISTA DE TECNOLOGIA DOS AGRONEGÓCIOS
INSTITUTO AGRONÔMICO

Pós-Graduação – Agricultura Tropical e Subtropical
Reconhecimento Homologado pela Portaria MEC Nº 609 de 14/03/2019 - D.O.U. 18/03/2019

ATA DE DEFESA DE DISSERTAÇÃO DE MESTRADO

Aos 31 de março de 2026, às 09h00, reuniu-se a banca examinadora homologada pelo Programa de Pós-Graduação em Agricultura Tropical e Subtropical, composta pelos membros abaixo listados visando à defesa de dissertação de mestrado de Stephanie Estete Pereira, para obtenção do título de "**MESTRE**", conforme Processo SAA nº PRT4266/2024-10. A sessão presidida pela Profª. Drª. Neidiquele Maria Silveira , orientadora da aluna, foi realizada em sessão pública aberta. Iniciados os trabalhos, a candidata submeteu-se ao exame de sua dissertação, intitulada "Desvendando o papel do óxido nítrico na tolerância à seca em plantas de citros". Terminado o exame, procedeu-se ao julgamento, cujo resultado foi o seguinte:

Profª. Drª. Neidiquele Maria Silveira - UNESP	**APROVADA (X)**	**REPROVADA ( )**
Prof. Dr. Fernando César Bachiega Zambrosi - IAC	**APROVADA (X)**	**REPROVADA ( )**
Profª. Drª. Marcela Trevenzoli Miranda - Unicamp	**APROVADA (X)**	**REPROVADA ( )**

Apurados os resultados, constatou-se que a candidata foi habilitada, fazendo jus, portanto, ao título de **"MESTRE EM AGRICULTURA TROPICAL E SUBTROPICAL"**, na área de concentração: Sistema de Manejo e Qualidade Ambiental, do que, para constar, lavrou-se a presente ata, assinada pelos membros da comissão examinadora:

Profª. Drª. Neidiquele Maria Silveira - UNESP

Prof. Dr. Fernando César Bachiega Zambrosi - IAC

Profª. Drª. Marcela Trevenzoli Miranda - Unicamp

AGRADECIMENTOS

A Deus, pelos sonhos dEle para mim, que são muito maiores que os meus.

Ao meu filho, Arthur Pereira Vallerio de Oliveira, minha maior motivação, agradeço pelo amor incondicional, pela paciência e por ser a razão do meu esforço diário.

Aos meus pais, Valdir Celso Pereira e Claudeci Santos Silva Pereira, mesmo diante das ausências e dificuldades, sempre estiveram ao meu lado, acreditando em mim e nos meus sonhos.

À minha amiga Ana Caroline dos Santos de Almeida, pelo companheirismo e por estar presente em todos os momentos ao longo desta trajetória. Os instantes de conversa, incentivo, troca e descontração foram essenciais para tornar esse caminho mais acolhedor.

À minha orientadora, Dra. Neidiquele Maria Silveira, pela orientação e pelos conhecimentos compartilhados. Sua dedicação e contribuição foram fundamentais.

Aos professores da PG-IAC pelos ensinamentos transmitidos, à secretaria do Programa de Pós-graduação e ao Instituto Agronômico pela oportunidade de realização do mestrado.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela concessão da bolsa de mestrado (processo nº 132594/2024-2), e à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, processo nº 2023/13662-3), pelo apoio financeiro ao desenvolvimento deste projeto.

À Universidade Estadual Paulista (UNESP), Campus Rio Claro, pelo espaço cedido para a realização do experimento, bem como pela infraestrutura disponibilizada.

Ao Centro de Citricultura Sylvio Moreira - IAC, pela disponibilização do espaço e da infraestrutura necessários para a realização de parte das análises moleculares. À Dra. Raquel Luciana Boscariol Camargo e ao Dr. Dilson Sousa Rocha Junior pela valiosa contribuição e auxílio na realização das análises de expressão de aquaporinas.

À Dra. Brenda Mistral de Oliveira Camargo, pelo apoio e auxílio nas análises de trocas gasosas e de potencial hídrico.

Aos membros da banca examinadora, Dr. Fernando Cesar Bachiega Zambrosi e Dra. Marcela Trevenzoli Miranda, pelas contribuições no exame de qualificação e na defesa, e à Dra. Brenda Mistral de Camargo Oliveira, pelas valiosas considerações na qualificação.

Aos colegas do grupo do Laboratório de Fisiologia Vegetal da UNESP, Jaqueline Cardili, Fernando Nakahara, em especial a Sabrina Henz, Maria Vitória Dainesi e Caio Menele, agradeço a colaboração e auxílio nas análises bioquímicas.
A todos aqueles que de forma direta ou indireta contribuíram para a realização desse trabalho. Muito Obrigada!

SUMÁRIO

INTRODUÇÃO GERAL

Eventos climáticos extremos, como ondas de calor e secas, provavelmente aumentarão em frequência e severidade em muitas áreas de cultivo, representando uma ameaça crescente aos ecossistemas naturais e agrícolas, especialmente quando ocorrem de forma simultânea como eventos combinados (Zscheischler et al., 2018). Nesse contexto, estudos envolvendo o limoeiro Cravo sob déficit hídrico buscam identificar os mecanismos que conferem resistência à seca a este porta-enxerto, o que representa um primeiro passo para o desenvolvimento de novos porta-enxertos cítricos capazes de garantir aos pomares um bom desempenho em períodos de escassez de água. Pesquisas mostram que plantas enxertadas em limoeiro Cravo são capazes de manter o crescimento radicular sob déficit hídrico (Pedroso et al., 2014). Além disso, o sistema radicular deste porta-enxerto apresenta alta condutividade hidráulica, o que confere melhor hidratação da parte aérea e manutenção da abertura estomática (Medina e Machado, 1998; Medina et al., 1998), favorecendo as trocas gasosas mesmo em condições de baixa disponibilidade hídrica. O limoeiro Cravo também tem a capacidade de redistribuir água internamente, o que foi vinculado ao ajuste osmótico e a regulação estomática sob déficit hídrico (Miranda et al., 2018, 2021). Recentemente, um estudo demonstrou que a sinalização química através de ABA evitou a desidratação em folhas do limoeiro Cravo sob déficit hídrico, sugerindo que a tolerância do limoeiro Cravo à seca é também baseada na sinalização química da baixa disponibilidade de água no solo (Miranda et al., 2022). A análise de expressão gênica em raízes de mudas de limoeiro Cravo cultivadas em condições hidropônicas com e sem déficit hídrico resultou na identificação de 40 genes diferencialmente expressos. Destes, foram identificados genes conhecidamente envolvidos na resposta ao estresse hídrico, como as aquaporinas (Boscariol-Camargo et al., 2007).

Um estudo recente analisou o perfil metabólico de porta-enxertos de limoeiro Cravo, citrumeleiro Swingle e tangerineira Sunki sob déficit hídrico. O limoeiro Cravo destacou-se pelo acúmulo de arginina nas folhas e raízes, especialmente após a reidratação, padrão não observado nos demais genótipos (Silva et al., 2023). A arginina, por apresentar alta razão N/C, atua como importante reserva e forma de transporte de nitrogênio orgânico, além de ser precursora do óxido nítrico (NO). Este, por sua vez, desempenha papel chave no desenvolvimento vegetal e na resposta a estresses abióticos, como a seca (Winter et al., 2015; Silveira et al., 2016; 2017a,b; 2019a; 2021). Em

condições de déficit hídrico, o NO promove o crescimento radicular e reduz danos oxidativos, atuando na eliminação de espécies reativas de oxigênio e estimulando enzimas antioxidantes como superóxido dismutase e catalase (Silveira et al., 2016; 2017b).

A produção de NO a partir da arginina em plantas ainda não é totalmente compreendida. Sabe-se que o NO pode ser sintetizado por vias dependentes de arginina ou de nitrito (Leitner et al., 2009; Fröhlich & Durner, 2011), embora os genes codificadores da enzima NO sintase (NOS) ainda não tenham sido identificados em plantas superiores (Foresi et al., 2010; Santolini et al., 2017; Astier et al., 2018; Hancock & Neill, 2019). Evidências bioquímicas, no entanto, indicam a conversão de arginina em NO e citrulina (Zhang et al., 2014; Domingos et al., 2015). O NO também pode formar *S*-nitrosoglutationa (GSNO) ao reagir com glutationa reduzida (GSH), participando de processos de S-nitrosação que modulam a atividade de proteínas (Fancy et al., 2017), como a Rubisco, cuja carboxilação é potencializada sob déficit hídrico (Silveira et al., 2017a, b).

De fato, as espécies e variedades de porta-enxertos apresentam raízes com características hidráulicas e anatômicas distintas, o que resulta em diferente capacidade de transportar água para a copa (Medina e Machado, 1998; Medina et al., 1998; Cohen e Naor, 2002; Martínez- Alcántara et al., 2013). Um dos fatores que podem influenciar a condutividade hidráulica das plantas é a abundância e a atividade de aquaporinas (Javot e Maurel, 2002), que são proteínas que facilitam o transporte de água e pequenas moléculas através de poros na bicamada fosfolipídica das membranas (Maurel et al., 2015). Este transporte transmembranar pode representar mais de 50% da condutividade hidráulica da raiz em espécies de plantas, como tomate, *Arabidopsis* e cana-de-açúcar (Maggio e Joly, 1995; Sutka et al., 2011; Silva et al., 2015). As aquaporinas são particularmente abundantes em plantas superiores, com mais de 30 isoformas (Gaspar, 2011). As proteínas intrínsecas da membrana plasmática (PIPs) são as principais aquaporinas envolvidas no transporte de água célula-célula e compõem até 20% da fração proteica das membranas plasmáticas. Em espécies cítricas, 11 PIPs foram encontradas em *Citrus clementina* e 8 PIPs em *Citrus sinensi*, no entanto, apenas quatro foram expressas em folhas (Martins et al., 2015). As PIPs ainda podem ser filogeneticamente classificadas em PIP1 e PIP2, sendo que as PIP2 apresentam maior capacidade de transporte de água (Chaumont et al., 2000). A co-expressão de PIP1 e PIP2 poderia aumentar a permeabilidade das membranas (Fetter et al., 2004; Yaneff et

al., 2015).

Alguns estudos têm relatado a participação do NO na indução da expressão de genes que codificam aquaporinas (Liu et al., 2007; Di Pietro et al., 2013). No entanto, o possível papel da sinalização de NO nas mudanças dos níveis de PIP em plantas sob déficit hídrico permanece desconhecido. Sabemos que o NO exógeno estimula a transcrição de *OsPIP1-1*, *OsPIP1-2*, *OsPIP1-3* e *OsPIP2-8* na germinação de sementes de arroz, sugerindo que os canais de água desempenham um papel importante na germinação, agindo, pelo menos em parte, em resposta à via de sinalização do NO (Liu et al., 2007). Adicionalmente, a análise proteômica abrangente de aquaporinas de raízes em *Arabidopsis* revelou que o NO exógeno modula a condutância hidraúlica da raiz em curto prazo (minutos), induzindo a inibição do transporte de água radicular (Di Pietro et al., 2013). Portanto, além das poucas evidências da participação do NO na expressão das aquaporinas, os estudos também são controversos.

Para compreender melhor o papel do NO nos processos fisiológicos, é comum o uso de sequestradores de NO endógeno, como o 2-(4-carboxifenil)-4,5-diidro-4,4,5,5-tetrametil-1H-imidazol-1-iloxi-3-óxido (cPTIO). O cPTIO é um radical orgânico estável desenvolvido por Akaike e Maeda (1996) e tem sido amplamente utilizado como ferramenta experimental, pois reage com o NO, oxidando-o e formando o radical NO_2. O uso do cPTIO permite avaliar a contribuição do NO endógeno em processos fisiológicos, incluindo a condutividade hidráulica e as respostas ao déficit hídrico. Conforme demonstrado por Pissolato (2020), a aplicação de cPTIO em plantas de cana-de-açúcar reduziu significativamente o acúmulo de NO tanto em folhas quanto em raízes, resultando em reduções na condutância estomática, na assimilação líquida de CO_2 e no crescimento radicular sob condições de déficit hídrico.

Diante desse contexto, e com o objetivo de aprofundar a compreensão sobre a contribuição do NO para a manutenção do status hídrico da parte aérea e para a modulação da expressão de aquaporinas em folhas e raízes de citros, esta dissertação foi estruturada em formato de artigo, conforme apresentado a seguir.

Referências

Akaike, T., & Maeda, H. (1996). Quantitation of nitric oxide using 2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO). In: Methods in Enzymology. Academic Press, p. 211-221. https://doi.org/10.1016/S0076-6879(96)68023-9.

Astier, J., Gross, I., & Durner, J. (2018). Nitric oxide production in plants: an update. Journal of Experimental Botany, 69: 3401-3411. https://doi.org/10.1093/jxb/erx420.

Boscariol-Camargo, R. L., Berger, I. J., Souza, A. A., Amaral, A. M. D., Carlos, E. F., Freitas-Astúa, J., Takita, M.A., Targon, M.L.P.N., Medina, C.L., Reis, M.S., Machado, M. A. (2007). In silico analysis of ESTs from roots of Rangpur lime (*Citrus limonia* Osbeck) under water stress. Genetics and Molecular Biology, 30: 906-916. https://doi.org/10.1590/S1415-47572007000500019.

Chaumont, F., Barrieu, F., Jung, R., & Chrispeels, M. J. (2000). Plasma membrane intrinsic proteins from maize cluster in two sequence subgroups with differential aquaporin activity. Plant Physiology 122: 1025-1034. https://doi.org/10.1104/pp.122.4.1025.

Cohen, S., Naor, A. (2002). The effect of three rootstocks on water use, canopy conductance and hydraulic parameters of apple trees and predicting canopy from hydraulic conductance. Plant, Cell and Environment, 25: 17–28, 2002. https://doi.org/10.1046/j.1365-3040.2002.00795.x.

de Paula Santos Martins C, Pedrosa AM, Du D, Gonçalves LP, Yu Q, Gmitter FG Jr, et al. (2015) Genome-Wide Characterization and Expression Analysis of Major Intrinsic Proteins during Abiotic and Biotic Stresses in Sweet Orange (*Citrus sinensis* L. Osb.). PLoS ONE 10(9): e0138786. https://doi.org/10.1371/journal.pone.0138786.

Di Pietro, M., Vialaret, J., Li, G. W., Hem, S., Prado, K., Rossignol, M., Maurel, C., & Santoni, V. (2013). Coordinated post- translational responses of aquaporins to abiotic and nutritional stimuli in Arabidopsis roots. Molecular & Cellular Proteomics, 12: 3886-3897, 2013.

Domingos, P., Prado, A. M., Wong, A., Gehring, C., & Feijo, J. A. (2015). Nitric oxide: a multitasked signaling gas in plants.Molecular Plant, 8: 506-520, 2015.

Fancy, N. N., Bahlmann, A. K., & Loake, G. J. (2017). Nitric oxide function in plant abiotic stress. Plant, Cell & Environment, 40: 462-472. https://doi.org/10.1111/pce.12707.

Fetter, K., Van Wilder, V., Moshelion, M., & Chaumont, F. (2004). Interactions between plasma membrane aquaporins modulate their water channel. The Plant Cell, 16: 215–228, 2004. https://doi.org/10.1105/tpc.017194.

Foresi, N., Correa-Aragunde, N., Parisi, G., Calo, G., Salerno, G., & Lamattina, L. (2010). Characterization of a nitric oxide synthase from the plant kingdom: NO generation from the green alga *Ostreococcus tauri* is light irradiance and growth phase dependent. Plant Cell, 22: 3816-3830, 2010. https://doi.org/10.1105/tpc.109.073510.

Fröhlich, A., & Durner, J. (2011). The hunt for plant nitric oxide synthase (NOS): is one really needed?. Plant Science, 181: 401-404. https://doi.org/10.1016/j.plantsci.2011.07.014.

Gaspar, M. (2011). Aquaporinas: de canais de água a transportadores multifuncionais em plantas. Brazilian Journal of Botany, 34: 481-491, 2011. https://doi.org/10.1590/S0100-84042011000400002.

Hancock, J. T., & Neill, S. J. (2019). Nitric oxide: its generation and interactions with other reactive signaling compounds. Plants, 8: 41. https://doi.org/10.3390/plants8020041.

Javot, H., & Maurel, C. (2002). The role of aquaporins in root water uptake. Annals of Botany, 90: 301-313, 2002. https://doi.org/10.1093/aob/mcf199.

Leitner, M., Vandelle, E., Gaupels, F., Bellin, D., & Delledonne, M. (2009). NO signals in the haze: nitric oxide signalling in plant defence. Current Opinion in plant biology, 12: 451-458, 2009. https://doi.org/10.1016/j.pbi.2009.05.012.

Liu, H. Y., Yu, X., Cui, D. Y., Sun, M. H., Sun, W. N., Tang, Z. C., .Kwak, S.S., & Su, W. A. (2007). The role of water channel proteins and nitric oxide signaling in rice seed germination. Cell research, 17: 638-649, 2007. https://doi.org/10.1038/cr.2007.34.

Maggio, A., & Joly, R. J. (1995). Effects of mercuric chloride on the hydraulic conductivity of tomato root systems (evidence for a channel-mediated water pathway). Plant Physiology. 109: 331-335. https://doi.org/10.1104/pp.109.1.331.

Martínez-Alcántara, B., Rodriguez-Gamir, J., Martínez-Cuenca, M. R., Iglesias, D. J., Primo-Millo, E., & Forner-Giner, M. A. (2013). Relationship between hydraulic conductance and citrus dwarfing by the Flying Dragon rootstock (*Poncirus trifoliata* L. Raft var. monstruosa). Trees-Structure and Function, 27: 629–638. https://doi.org/10.1007/s00468-012-0817-1.

Maurel, C., Boursiac, Y., Luu, D. T., Santoni, V., Shahzad, Z., & Verdoucq, L. (2015). Aquaporins in plants. .Physiological Reviews, 95: 1321–1358, 2015. https://doi.org/10.1152/physrev.00008.2015.

Medina, C. L., & Machado, E. C. (1998). Gas exchange and water relations of "Valencia" orange tree grafted on "Rangpur" lime and *Poncirus trifoliata*, submitted to a water deficit. Bragantia (Brazil), *57*(1):15-22.

Medina, C. L., Machado, E. C., & Pinto, J. M. (1998). Fotossíntese de laranjeira'Valência'enxertada sobre quatro porta-enxertos e submetida à deficiência hídrica. Bragantia, 57: 1-14. https://doi.org/10.1590/S0006-87051998000100001.

Miranda, M. T., da Silva, S. F., Moura, B. B., Hayashi, A. H., Machado, E. C., & Ribeiro, R. V. (2018). Hydraulic redistribution in Citrus rootstocks under drought. Theoretical and Experimental Plant Physiology, 30: 165-172, 2018. https://doi.org/10.1007/s40626-018-0111-8.

Miranda, M. T., Da Silva, S. F., Silveira, N. M., Pereira, L., Machado, E. C., & Ribeiro, R. V. (2021). Root osmotic adjustment and stomatal control of leaf gas exchange are dependent on citrus rootstocks under water deficit. Journal of Plant Growth Regulation, 40: 11-19. https://doi.org/10.1007/s00344-020-10069-5.

Miranda, M. T., Espinoza-Núñez, E., Silva, S. F., Pereira, L., Hayashi, A. H., Boscariol-Camargo, R. L., Carvalho, S.A., Machado, E.C., Ribeiro, R. V. (2022). Water stress signaling and hydraulic traits in three congeneric citrus species under water deficit. Plant Science, 319: p. 111255. https://doi.org/10.1016/j.plantsci.2022.111255.

Pedroso, F. K., Prudente, D. A., Bueno, A. C. R., Machado, E. C., & Ribeiro, R. V. (2014). Drought tolerance in citrus trees is enhanced by rootstock-dependent changes in root growth and carbohydrate availability. Environmental and Experimental Botany, 101: 26-35. https://doi.org/10.1016/j.envexpbot.2013.12.024.

Pissolato, M. D., Silveira, N. M., Prataviera, P. J. C., Machado, E. C., Seabra, A. B., Pelegrino, M. T., Sodek, L., Ribeiro, R. V. (2020). Enhanced nitric oxide synthesis through nitrate supply improves drought tolerance of sugarcane plants. Frontiers in Plant Science, 11: 970, 2020. https://doi.org/10.3389/fpls.2020.00970.

Santolini, J., André, F., Jeandroz, S., & Wendehenne, D. (2017). Nitric oxide synthase in plants: where do we stand?. Nitric Oxide, 63: 30-38. https://doi.org/10.1016/j.niox.2016.09.005.

Silva, K. I., Sales, C. R., Marchiori, P. E., Silveira, N. M., Machado, E. C., & Ribeiro, R. V. (2015). Short-term physiological changes in roots and leaves of sugarcane varieties exposed to H_2O_2 in root medium. Journal of plant physiology, 177: 93-99. https://doi.org/10.1016/j.jplph.2015.01.009.

Silva, S. F., Miranda, M. T., Cunha, C. P., Domingues-Jr, A. P., Aricetti, J. A., Caldana, C., Machado, E.C., & Ribeiro, R. V. (2023). Metabolic profiling of drought tolerance: revealing how citrus rootstocks modulate plant metabolism under varying water availability. Environmental and Experimental Botany, 206: 105169. https://doi.org/10.1016/j.envexpbot.2022.105169.

Silveira, N. M., Frungillo, L., Marcos, F. C., Pelegrino, M. T., Miranda, M. T., Seabra, A. B., Salgado, I., Machado, E.C., Ribeiro, R. V. (2016). Exogenous nitric oxide improves sugarcane growth and photosynthesis under water deficit. Planta, 244: 181-190. https://doi.org/10.1007/s00425-016-2501-y.

Silveira, N. M., Hancock, J. T., Frungillo, L., Siasou, E., Marcos, F. C., Salgado, I., Machado, E.C., Ribeiro, R. V. (2017a). Evidence towards the involvement of nitric oxide in drought tolerance of sugarcane. Plant physiology and biochemistry, 115: 354-359. https://doi.org/10.1016/j.plaphy.2017.04.011.

Silveira, N. M., Marcos, F. C., Frungillo, L., Moura, B. B., Seabra, A. B., Salgado, I., Machado, E.C., Hancock, J.T., Ribeiro, R. V. (2017b). S-nitrosoglutathione spraying improves stomatal conductance, Rubisco activity and antioxidant defense in both leaves and roots of sugarcane plants under water deficit. Physiologia plantarum, 160(4): 383-395. https://doi.org/10.1111/ppl.12575.

Silveira, N. M., Seabra, A. B., Machado, E. C., Hancock, J. T., & Ribeiro, R. V. (2019a). Nitric Oxide-Induced Tolerance in Plants under Adverse Environmental Conditions. In Approaches for Enhancing Abiotic Stress Tolerance in Plants (pp. 371-386). CRC Press.

Silveira, N. M., Ribeiro, R. V., de Morais, S. F., de Souza, S. C., da Silva, S. F., Seabra, A. B., Hancock, J.T., Machado, E. C. (2021). Leaf arginine spraying improves leaf gas exchange under water deficit and root antioxidant responses during the recovery period. Plant Physiology and Biochemistry, 162: 315-326. https://doi.org/10.1016/j.plaphy.2021.02.036.

Sutka, M., Li, G., Boudet, J., Boursiac, Y., Doumas, P., & Maurel, C. (2011). Natural variation of root hydraulics in Arabidopsis grown in normal and salt-stressed conditions. Plant physiology, 155(3): 1264-1276. https://doi.org/10.1104/pp.110.163113.

Winter, G., Todd, C. D., Trovato, M., Forlani, G., & Funck, D. (2015). Physiological implications of arginine metabolism in plants. Frontiers in plant science, *6*: 534.https://doi.org/10.3389/fpls.2015.00534.

Yaneff, A., Vitali, V., & Amodeo, G. (2015). PIP1 aquaporins: Intrinsic water channels or PIP2 aquaporin modulators?. FEBS letters, *589*(23), 3508-3515. https://doi.org/10.1016/j.febslet.2015.10.018.

Zhang, J. J., Li, X. Q., Sun, J. W., & Jin, S. H. (2014). Nitric oxide functions as a signal in ultraviolet-B-induced baicalin accumulation in Scutellaria baicalensis suspension cultures. International journal of molecular sciences, *15*(3): 4733-4746. https://doi.org/10.3390/ijms15034733.

Zscheischler, J., Westra, S., Van Den Hurk, B. J., Seneviratne, S. I., Ward, P. J., Pitman, A., ... & Zhang, X. (2018). Future climate risk from compound events. Nature climate change, 8(6), 469-477. https://doi.org/10.1038/s41558-018-0156-3

Manuscrito

Endogenous nitric oxide differentially modulates aquaporin expression in leaves and roots and redox homeostasis in citrus.

Abstract

This study evaluated the role of endogenous nitric oxide (NO) in drought tolerance in 'Rangpur' lime plants, focusing on its contribution to shoot water status maintenance and the modulation of aquaporin expression in leaves and roots. We hypothesized that NO regulates aquaporin expression, thereby influencing plant hydraulic balance and mitigating oxidative damage. Treatments consisted of well-watered plants (control), control with the addition of cPTIO (an endogenous NO scavenger), water deficit (WD), and WD combined with cPTIO. Water deficit was induced by adding polyethylene glycol (PEG-8000) to the nutrient solution. Aquaporin gene expression, leaf water potential, physiological traits, biomass, and oxidative stress markers were assessed. Overall, endogenous NO modulated aquaporin expression in an organ-specific manner. Under water deficit, NO removal by cPTIO increased PIP2.1 expression in leaves and was associated with higher leaf hydration. In contrast, in roots, cPTIO reduced PIP1 expression under drought conditions. NO also influenced shoot hydration in a condition-dependent manner, as it contributed to higher leaf water status under adequate irrigation but was associated with reduced hydration under drought. Together, these findings demonstrate that the role of NO extends beyond stress signaling, contributing to the coordinated regulation of water transport and redox homeostasis in citrus plants.

Keywords: Water transport; oxidative stress; PIP1; PIP2.1; water deficit.

1. Introduction

‘Rangpur’ lime is recognized as a drought-tolerant citrus rootstock, exhibiting physiological mechanisms that contribute to the maintenance of shoot hydration under low soil water availability. Previous studies have shown that plants grafted onto this rootstock are able to sustain root growth and high root hydraulic conductivity, thereby promoting stomatal opening and gas exchange even under water deficit conditions (Medina & Machado, 1998; Medina et al., 1998; Pedroso et al., 2014). In addition, drought tolerance in ‘Rangpur’ lime has been associated with enhanced internal water redistribution, ABA-mediated chemical signaling, and the formation of thicker pit membranes in xylem, which confer greater embolism resistance compared with other rootstocks (Miranda et al., 2024).

Recent evidence indicates that, in addition to abscisic acid (ABA), nitric oxide (NO) plays a relevant role in the drought tolerance of ‘Rangpur’ lime. We demonstrated that ‘Rangpur’ lime exhibits higher NO production in roots and superior root growth compared with ‘Swingle’ citrumelo under low water availability (Silveira et al., 2024). Notably, the role of NO extends beyond the modulation of root biomass accumulation, as it also influences root morphology. Elevated NO levels are associated with significant increases in root length and root surface area (Silveira et al., 2024). In addition, NO acts as a signaling molecule that mitigates oxidative damage in plants subjected to water deficit by promoting superoxide anion scavenging and enhancing the activity of antioxidant enzymes such as superoxide dismutase and catalase (Silveira et al., 2017b).

Nitric oxide (NO) production in plants remains not fully understood, in contrast to mammals, where it is well characterized. In plants, the main known pathway involves the activity of nitrate reductase (NR), in addition to NO production from nitrite via the mitochondrial electron transport chain (Kumari et al., 2023) and through non-enzymatic routes (Kolbert et al., 2019). There is also evidence for oxidative pathways similar to those described in mammals, with the detection of “nitric oxide synthase (NOS)-like” activity in peroxisomes and chloroplasts. However, no homologs of NOS genes have been identified in plants (Jeandroz et al., 2016), suggesting the existence of structurally distinct and yet unidentified enzymes. Furthermore, polyamines have been proposed as potential sources of NO, although conclusive evidence supporting this mechanism is still lacking (Corpas and Barroso, 2017). Supporting the role of NO in the root system under drought, gene expression analyses in roots of ‘Rangpur’ lime seedlings revealed approximately 40

differentially expressed genes, including genes classically associated with plant responses to water stress, such as aquaporins (Boscariol-Camargo et al., 2007). In this context, root hydraulic conductivity is strongly influenced by the abundance and activity of aquaporins, particularly plasma membrane intrinsic proteins (PIPs), which play a fundamental role in cell-to-cell water transport (Javot & Maurel, 2002; Maurel et al., 2015). Although some studies suggest that NO may modulate the expression of aquaporin-encoding genes (Liu et al., 2007; Di Pietro et al., 2013), the available evidence remains limited and, in some cases, contradictory, especially under water deficit conditions. For instance, exogenous NO has been shown to induce aquaporin gene transcription during rice seed germination (Liu et al., 2007), whereas proteomic analyses in *Arabidopsis* indicate that NO rapidly inhibits root hydraulic conductance by reducing root water transport (Di Pietro et al., 2013). Therefore, although NO appears to play a role in aquaporin regulation, its involvement in PIP expression under water deficit conditions remains uncertain.

In studies investigating NO-mediated processes, the NO scavenger cPTIO is commonly employed to specifically inhibit endogenous NO activity, enabling the assessment of its contribution to diverse physiological responses. Thus, cPTIO represents a valuable tool for elucidating NO-dependent mechanisms in plants under different experimental conditions. In this context, the aim of this study was to evaluate the role of endogenous NO in drought tolerance in 'Rangpur' lime plants, with emphasis on its contribution to the maintenance of shoot water status and the modulation of aquaporin expression in leaves and roots. We hypothesized that NO modulates aquaporin expression, thereby contributing to the maintenance of shoot water status and mitigating oxidative damage through the regulation of the antioxidant system.

2. Material and methods

2.1 Plant material and experimental conditions

Valencia sweet orange plants (*Citrus sinensis* (L.) Osbeck) grafted onto 'Rangpur' lime (*Citrus limonia* Osbeck) rootstock were used in this study. The plants were approximately 10 months old at the beginning of the experiment. 'Rangpur' lime is a species generally used as rootstock because of its superior agronomic performance under water deficit (Cimen and Yesiloglu, 2016; Ribeiro et al., 2014).

Prior to the treatments, the plants were carefully washed with deionized water to remove adhered substrate and subsequently transferred to nutrient solution. Plants were grown in plastic boxes (22L), containing a nutrient solution following the Hoagland and Arnon (1950), The pH of nutrient solution was kept between 5.5 and 6.0 and its electrical conductivity between 1.8 and 2.0 mS cm^{-1} by daily monitoring. The solution volume was constantly monitored and aeration was performed with an air compressor.

Plants were maintained under greenhouse conditions throughout the experimental period, where mean air temperature and relative humidity varied between 27 ºC and 36 ºC and 57%, respectively. The plants were acclimated for a period of six days, until the emergence of new sprouts. After this period, only three shoots were maintained per plant.

2.2 Imposition of treatments: water deficit and application of the NO scavenger

After the acclimation period, the experimental treatments were initiated as follows: (a) control, nutrient solution with an osmotic potential of −0.15 MPa; (b) control with foliar application of cPTIO (100 µM) (Pissolato et al., 2020); (c) water deficit (WD), nutrient solution adjusted to an osmotic potential of -0.4 MPa; and (d) WD with cPTIO (100 µM). The osmotic potential of -0.4 MPa was selected based on previous studies with citrus plants, which reported a significant reduction in accumulated dry mass under this condition (Girardi et al., 2018). To minimize osmotic shock, the reduction in osmotic potential was applied progressively, reaching -0.25 MPa on the first day, -0.3 MPa on the second day, and -0.4 MPa on the third day, corresponding to the target level for water deficit treatment.

For treatments (b) and (d) were provided a NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline1-oxyl-3-oxide (cPTIO). cPTIO is a stable organic radical developed by Akaike and Maeda (1996), which has been widely used as a control as it oxidizes the NO molecule to form NO_2. For the application of cPTIO, the plants were transferred and the roots placed in a humid box, where they were sprayed 50 ml with cPTIO the root system of each plant, and it remained in the dark for 1 hour. After the treatment, the plants were returned to the boxes with their respective solutions. (Pissolato et al., 2020). This procedure was carried out for four consecutive days from the moment the water deficit (-0.4 MPa) was established.

One day after the last cPTIO application, leaf and root samples were collected and immediately immersed in liquid nitrogen and stored at -80 °C for analysis of relative

expression of aquaporins, ROS and antioxidant enzyme activities. At the end of the experiment (8th day), measurements of gas exchange, leaf water potential, chlorophyll content, and biometry were performed (Fig. 1).

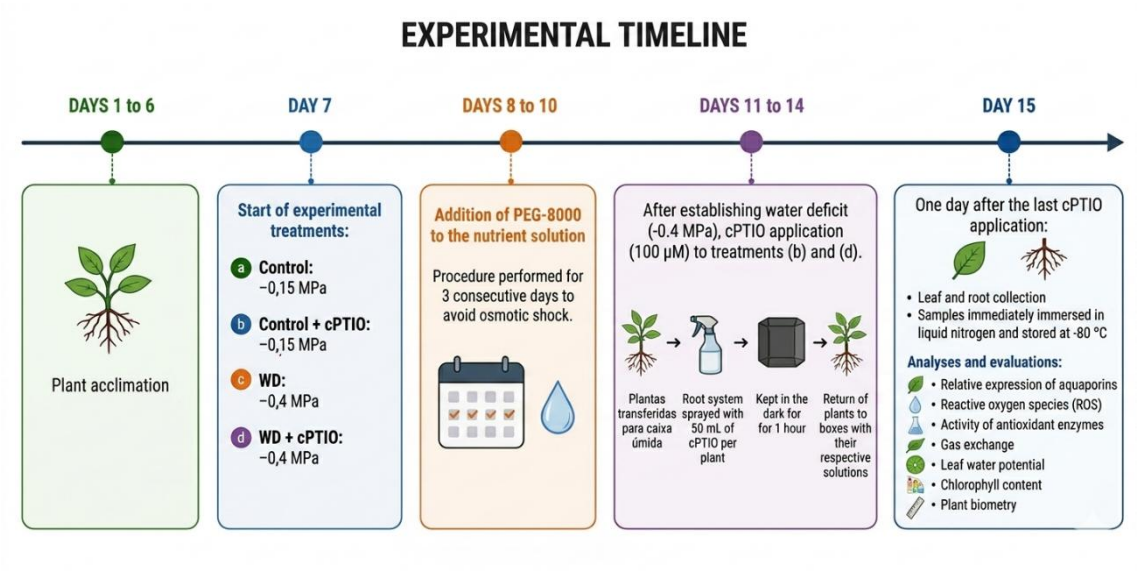

Figure 1. Simplified schematic diagram illustrating the experimental design, including the imposition of water deficit using polyethylene glycol (PEG), cPTIO application, and sampling procedures. Abbreviations: WD, water deficit; PEG, polyethylene glycol; cPTIO, nitric oxide (NO) scavenger. Source: Gemini artificial intelligence (Google, 2026).

2.3 Leaf water potential and biometry

Leaf water potential (Ψ_w) was measured between 10:00 and 12:00 h in fully expanded leaves using a pressure chamber (Model 3005F01 Plant Water Status Console; Soil Moisture Equipment Corp., Santa Barbara, CA, USA). At the end of experiment, leaves and roots were harvested, and the dry matter quantified after drying samples in an oven (60°C) with forced-air circulation until constant weight. The leaf area of each plant was evaluated with a portable leaf area meter (LI-3100C, Li-Cor Inc., Lincoln NE, USA), following the manufacturer's instructions.

2.4 Analysis of aquaporin gene expression by quantitative real-time PCR (RT-qPCR)

Total RNA from leaves and roots was isolated using QIAzol® reagent, following the manufacturer's instructions. The quality and integrity of the isolated RNA were assessed on a 1% agarose gel and quantified using NANOdrop (Thermo Scientific™, 2000/2000c, Wilmington, USA). Contaminating DNA was treated with DNase I (Promega Corporation, USA). After quantification and cDNA synthesis (Promega Corporation kit, USA), gene expression was analyzed by quantitative real-time PCR (RT-qPCR) using an Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) and a Master Mix GoTaq detection system (Promega Corporation, USA). Each run consisted of three biological replicates per treatment with three technical replicates in each. GAPC2 was used as a reference gene in root analysis and EF-1α was used in leaf samples, following Mafra et al. (2012).

The expression of PIP1, PIP2.1 and PIP2.5 genes in roots and leaves were evaluated by relative quantification based on the 2-ΔΔCt method (Livak and Schmittgen, 2001).

2.5 Leaf gas exchange, photochemistry and chlorophyll content

The CO_2 assimilation (A) and stomatal conductance (g_s) were measured using an infrared gas analyzer (LI-6400, LI-COR Inc., Lincoln, NE, EUA). Measurements were performed between 10:00 and 12:30 h under photosynthetically active radiation of 1000 μmol m^{-2} s^{-1}, ambient temperature and non-atmospheric CO_2 concentration of 400 μmol mol^{-1}. The instantaneous carboxylation efficiency (k = A/Ci) was calculated according to Machado et al. (2009). Chlorophyll fluorescence was evaluated simultaneously to leaf gas exchange and the effective quantum efficiency of photosystem II (Φ_{PSII}) was estimated by the saturation pulse method (Edwards and Baker, 1993). For the estimation of chlorophyll content, a portable chlorophyll meter (CFL 2060, Falker, Porto Alegre, RS, Brazil) was used to evaluate chlorophyll *a* and *b* contents, and the relative content of total chlorophyll was calculated as chlorophyll *a* + *b*.

2.6 Hydrogen peroxide and lipid peroxidation

Frozen leaves and roots (0.2 g) were homogenized in 0.1% (w/v) trichloroacetic acid and centrifuged at 13,000 × g at 4°C for 20 min. Then, H_2O_2 was quantified as described by Velikova et al. (2000). Aliquots of 300 µl of supernatant were added to the

reaction medium containing 10 mM phosphate buffer (pH 7.0) and 1 M potassium iodide. The samples were incubated at 30°C for 30 min and the absorbances were determined at 390 nm. H_2O_2 concentration was estimated based on a calibration curve. Another supernatant aliquot (300 µl) was also incubated in a reaction medium containing 0.5% thiobarbituric acid (w/v) and 10% (w/v) trichloroacetic acid at 90°C. After 20 min, the reaction was stopped in an ice bath for 10 min, absorbance readings were taken at 535 and 600 nm and malondialdehyde (MDA) concentration was calculated as described by Cakmak and Horst (1991). The MDA concentration was used as a proxy of lipid peroxidation.

2.7 Antioxidant activity

Leaves and roots (0.2 g) were homogenized in a 100 mM phosphate buffer (pH 7.8) containing 100 µM EDTA, 10 mM ascorbic acid, and 10% (w/w) polyvinylpolypyrrolidone. The extracts were centrifuged at 12,000 × g for 30 min at 4°C, and the resulting supernatants were used as the crude enzymatic extract. Protein content was quantified using the Bradford method (Bradford 1976), with bovine serum albumin (BSA) as the standard.

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was determined by monitoring the inhibition of nitroblue tetrazolium photoreduction at 560 nm (Giannopolitis and Ries 1977). The SOD reaction medium consisted of 50 mM phosphate buffer (pH 7.8), 14 mM methionine, 0.1 µM EDTA, 75 µM nitroblue tetrazolium, and 2.0 µM riboflavin (Giannopolitis and Ries 1977).

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured by following ascorbate oxidation in a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, and 0.1 mM H_2O_2. The decrease in absorbance at 290 nm was monitored, and enzyme activity was calculated using the molar extinction coefficient of ascorbate of 2.8 mM^{-1} cm^{-1} and expressed as µmol min^{-1} mg^{-1} of protein (Nakano and Asada 1981).

Catalase (CAT; EC 1.11.1.6) activity was evaluated by incubating the enzymatic extracts in a reaction mixture containing 200 mM phosphate buffer (pH 7.0) and 12.5 mM H_2O_2. The decrease in absorbance at 240 nm was monitored, and enzyme activity was calculated according to the molar extinction coefficient of H_2O_2 (36 mM^{-1} cm^{-1}) and expressed as µmol min^{-1} mg^{-1} of protein (Azevedo et al. 1998).

2.8 Data analysis

Data were analyzed using Bayesian statistics (JASP software, https://jaspstats.org/). When significant differences were detected, the mean values were compared using Bayes Factor (BF_{10}): $1 < BF_{10} < 3$, there is a weak support for the alternative hypothesis (H1); $3 < BF_{10} < 20$ indicates positive support for H1; and $BF_{10} > 20$ indicates strong support to H1 (Miranda et al., 2021). The results presented are the mean ± standard error and the number of replicates is stated in each figure caption.

3. Results

3.1 Leaf water potential, plant biomass and leaf gas exchange

Leaf water potential decreased in plants subjected to water deficit compared with well-hydrated treatments (control and control + cPTIO). The addition of the NO scavenger cPTIO resulted in distinct effects depending on water availability. Under well-hydrated conditions, cPTIO application reduced leaf water potential compared with control plants. In contrast, under water deficit, cPTIO application resulted in a higher leaf hydration (Fig. 2A). Regarding leaf and root dry mass, the removal of endogenous NO through cPTIO application led to a reduction in leaf dry mass under both water conditions (Fig. 2B). In contrast, no significant differences in root dry mass were observed among treatments, regardless of water availability (Fig. 2C).

Photosynthetic rate (A), as well as diffusive (g_s), photochemical (Φ_{PSII}), and biochemical (k) parameters, were not affected by either water availability or cPTIO application. Similarly, leaf area and total chlorophyll content did not differ among treatments (Fig. 3).

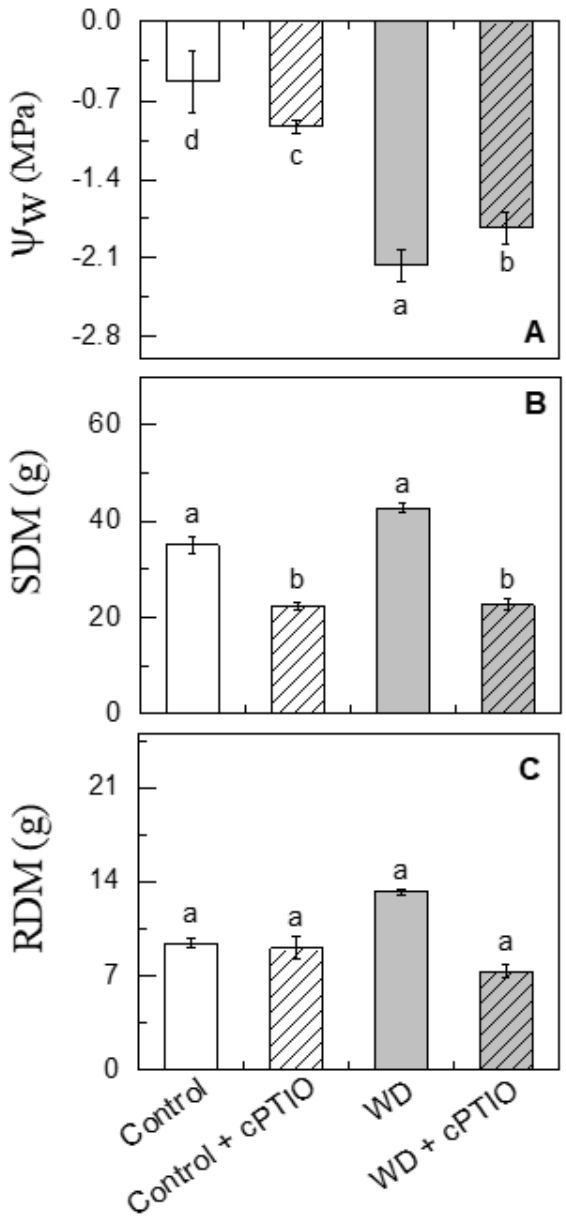

Figure 2. Leaf water potential (ψ_w, in A), shoot (SDM, in B) and root dry mass (RDM, in C) of Valencia orange scions grafted on 'Rangpur' lime, under well-hydrated conditions (control), control + cPTIO, water deficit (WD) and WD + cPTIO. Measurements were taken at the end of the experiment (14th day). Data represent the mean values of three replicates ± standard error. Letters indicate statistical difference (BF_{10} > 3) between treatments.

3.2 Aquaporin expression

In leaves, the addition of the NO scavenger cPTIO resulted in higher expression of PIP2.1 compared with the WD treatment, whereas PIP1 and PIP2.5 expression did not differ with cPTIO application (Fig. 4A). In roots, cPTIO application resulted in lower expression of PIP1 compared with the WD treatment, while PIP2.1 and PIP2.5 expression were not affected by cPTIO application (Fig. 4B).

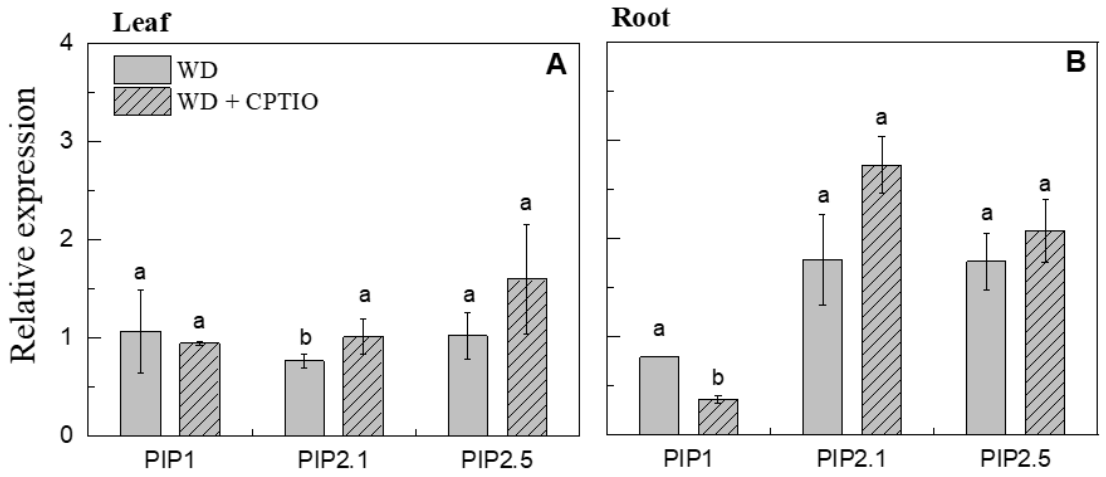

Figure 4. Relative expression of the aquaporin genes PIP1, PIP2.1 and PIP2.5 in leaves and roots of Valencia orange scions grafted on 'Rangpur' lime, under water deficit (WD) and WD + cPTIO. Gene expression was quantified by RT-qPCR and normalized by the $2^{-}\Delta\Delta Ct$ method and by control treatment. The data represent the mean values of three replicates ± standard error. Letters indicate statistical difference ($BF_{10} > 3$) between treatments.

3.3 Oxidative damage and antioxidant metabolism

In leaves, the addition of the NO scavenger cPTIO increased MDA concentration in well-watered plants, whereas under water deficit, MDA levels were higher compared with the control, regardless of cPTIO application (Fig. 5A). In roots, MDA concentration was higher under water deficit compared with the control, however, with no differences associated with cPTIO application within each water condition (Fig. 5B). Regarding H_2O_2 concentration, no differences were observed among treatments in both leaves and roots, regardless of water condition or cPTIO application (Fig. 5C, D).

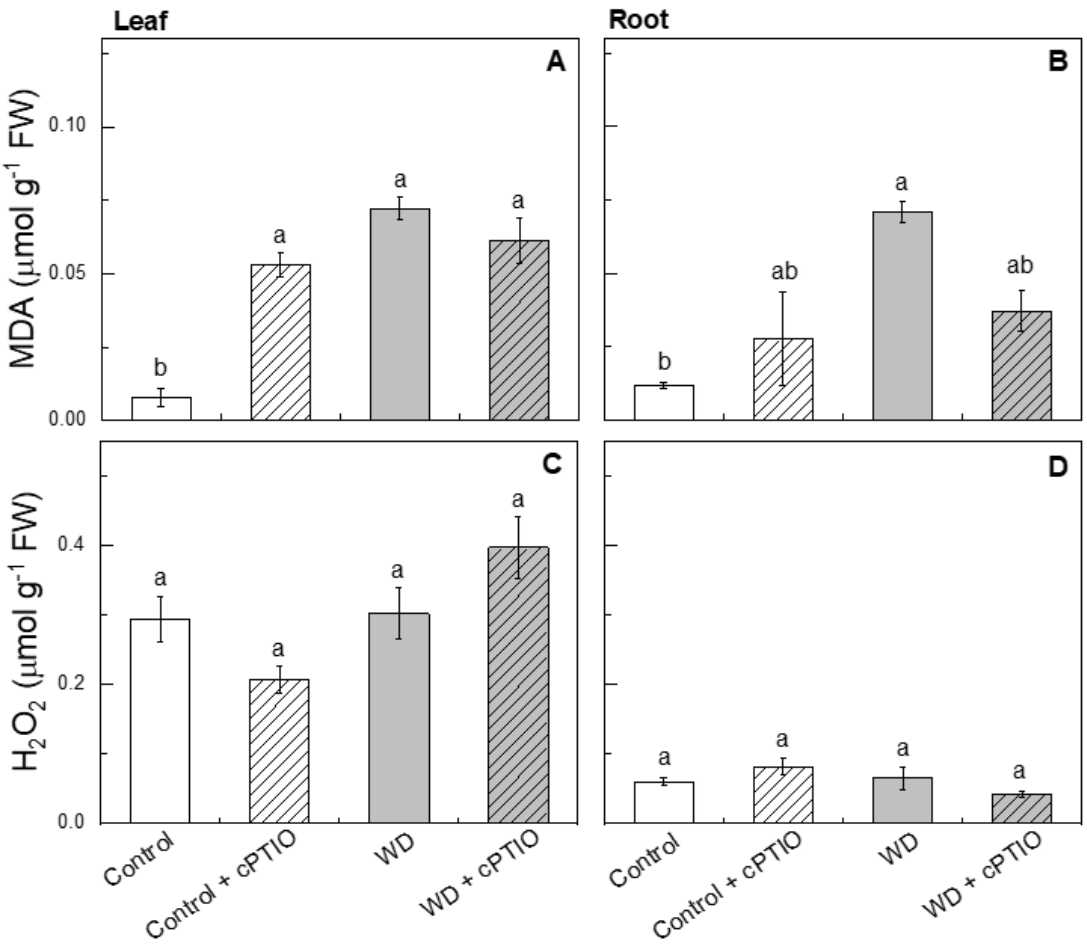

Figure 5. Concentration of malondialdehyde (MDA, in A and B) and hydrogen peroxide (H_2O_2, in C and D) in leaves (A, C) and roots (B, D) of Valencia orange scions grafted on 'Rangpur' lime, under well-hydrated conditions (control), control + cPTIO, water deficit (WD) and WD + cPTIO. The data represent the mean values of three replicates ± standard error. Letters indicate statistical difference ($BF_{10} > 3$) between treatments.

Regarding antioxidant metabolism, root CAT activity was reduced in plants treated with the NO scavenger cPTIO under both well-watered and water deficit conditions (Fig. 6B). There were no differences observed among treatments in leaf CAT activity and in APX and SOD activities in either leaves or roots, regardless of water availability or cPTIO application (Fig. 6A, C–F).

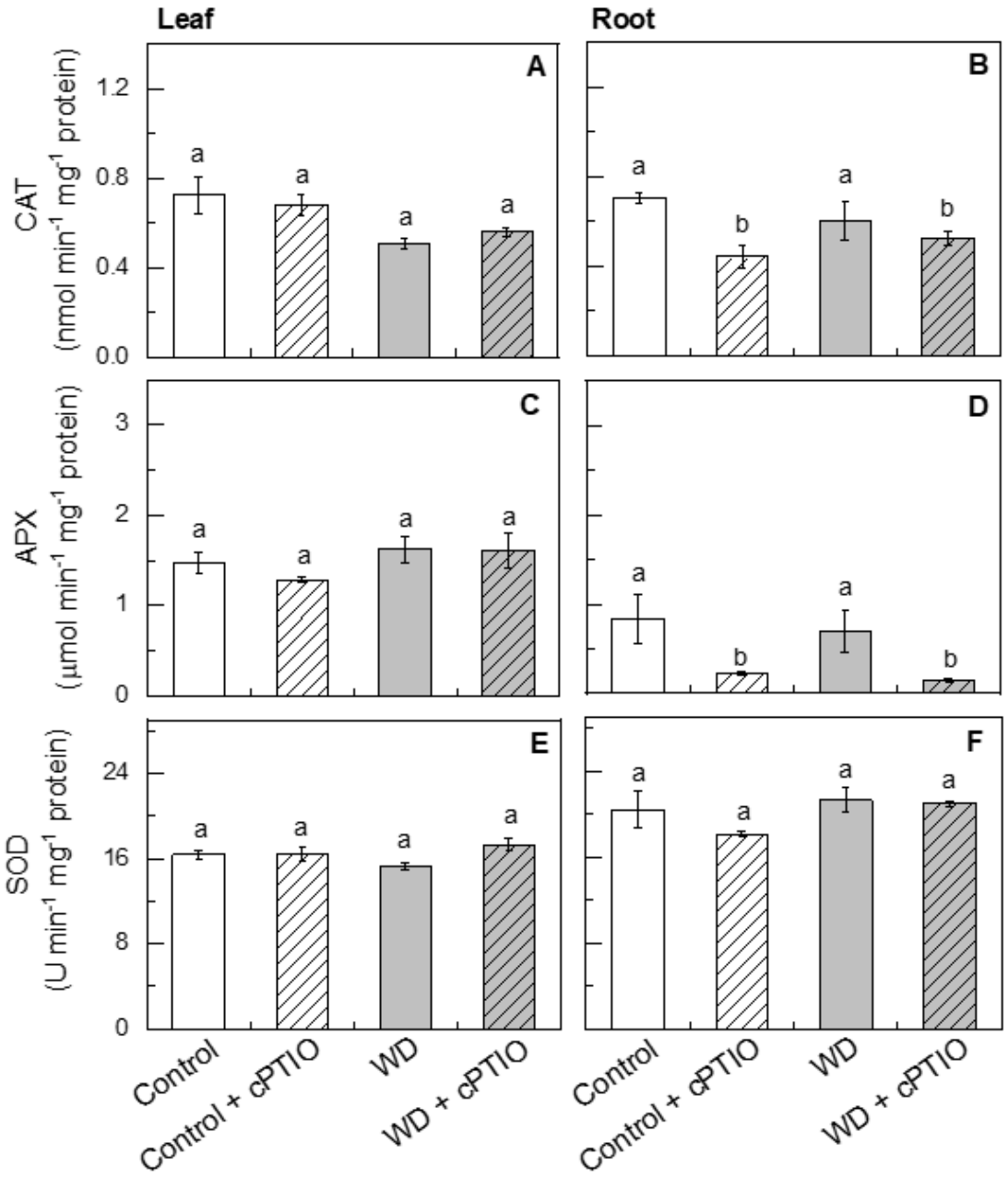

Figure 6. Activity of catalase (CAT, in A and B), ascorbate peroxidase (APX, in C and D) and superoxide dismutase (SOD, in E and F) in leaves (A, C, E) and roots (B, D, F) of Valencia orange scions grafted on 'Rangpur' lime, under well-hydrated conditions (control), control + cPTIO, water deficit (WD) and WD+ cPTIO. The data represent the mean values of three replicates ± standard error. Letters indicate statistical difference ($BF_{10} > 3$) between treatments.

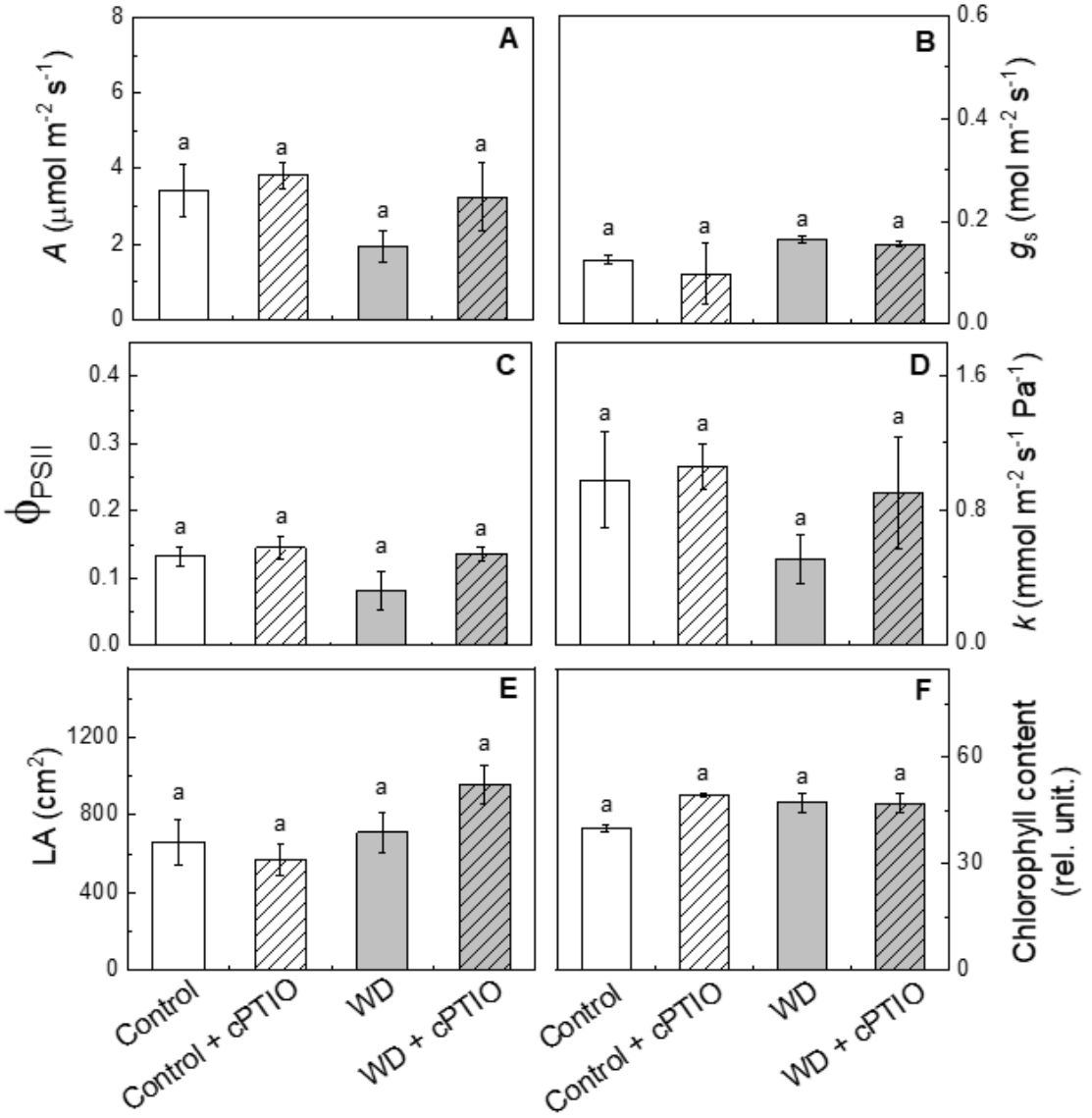

Figure 3. Foliar CO_2 assimilation (A, in A), stomatal conductance (g_s, in B), effective quantum efficiency of photosystem II (ϕ_{PSII}, in C), instantaneous carboxylation efficiency (k, in D), Leaf area (LA, in E) and total chlorophyll (relative units, in F) of Valencia orange scions grafted on 'Rangpur' lime, under well-hydrated conditions (control), control + cPTIO, water deficit (WD) and WD + cPTIO. The data represent the mean values of three replicates ± standard error. Letters indicate statistical difference (BF_{10} > 3) between treatments.

4. Discussion

4.1 Nitric oxide modulates aquaporin expression in leaves and roots in a different manner

Our results indicate that endogenous NO modulates aquaporin expression in an organ-specific manner. Under drought, the removal of endogenous NO by cPTIO application increased PIP2.1 expression in leaves, which likely enhanced membrane water permeability and contributed to the higher leaf hydration (Figs. 2A and 3A) This response suggests that, in leaves, NO may act as a negative regulator of aquaporin expression. In contrast, in roots, cPTIO treatment reduced PIP1 expression under water deficit, indicating that endogenous NO is required to sustain the expression of specific aquaporins involved in root water uptake and radial water transport under water deficit condition.

In fact, several studies have demonstrated that the overexpression of the aquaporin gene PIP1 is beneficial for plants under stress conditions (Ayadi et al., 2014; Hu et al., 2012). For example, enhanced PIP1 expression increased salt stress tolerance in *Triticum turgidum*, resulting in improved plant growth, higher relative water content, and reduced membrane damage under saline conditions. Moreover, transgenic plants exhibited lower levels of lipid peroxidation, indicating reduced oxidative stress (Hu et al., 2012). Additionally, Miranda et al. (2022) suggested that the 'Rangpur' lime rootstock exhibits a rapid drought-response mechanism characterized by stomatal closure associated with the downregulation of PIP2.1 and PIP2.5 in leaves, which partially supports our findings, as we also detected reduced PIP2.1 expression under water deficit conditions (Fig. 4A).

In this context, NO appears to act as an important signaling molecule, coordinating hydraulic adjustments through the modulation of aquaporin gene expression. In addition, we observed a reduction in leaf water potential under well-watered conditions following the use of the NO scavenger cPTIO (Fig. 2A), indicating that endogenous NO plays a constitutive role in maintaining hydraulic homeostasis. These findings suggest that NO fine-tunes basal water transport processes even in the absence of environmental stress. Although no significant differences in gas exchange were detected among treatments in this study (Fig. S1), previous studies have shown that NO can promote stomatal closure in coordination with ABA signaling (Desikan et al., 2004; Bright et al., 2006), which may explain the higher leaf water status observed in plants with endogenous NO under well-watered conditions.

In contrast, under drought conditions, NO was associated with lower leaf water potential (Fig. 2A). Indeed, studies have demonstrated that exogenous NO can partially maintain stomatal opening and CO_2 assimilation under restrictive conditions (Silveira et al., 2016, 2017a, 2021a,b), which may this reduced leaf water potential observed under water deficit. The role of NO in stomatal regulation is highly context-dependent and modulated by its interaction with other signaling molecules, likely explaining its contrasting effects on plant water status under different water regimes.

In addition, NO removal through cPTIO application reduced leaf dry mass under both water conditions (Fig. 2B). Although no significant changes in leaf area were detected (Fig. S1E), NO may influence leaf structural traits, such as mesophyll thickness, thereby affecting leaf density (Zheng et al., 2021). Indeed, exogenous NO application under aluminum stress increased mesophyll thickness and altered palisade and spongy tissue organization in watermelon leaves, suggesting a direct effect on foliar structural traits (Zheng et al., 2021). These structural adjustments may have important implications for leaf mechanical stability and functional longevity. In perennial crops such as citrus, this type of regulation is particularly relevant, as the maintenance of leaf structural integrity throughout prolonged growth cycles depends on a fine balance between redox signaling, metabolism, and cell wall remodeling. In this context, the results obtained in the present study reinforce the importance of NO not only in stress responses but also in preserving leaf functionality and structural stability during plant development.

4.2 Endogenous nitric oxide as a key regulator of redox homeostasis

Our results showed an increase in malondialdehyde (MDA) concentration in the leaves of well-watered plants following NO removal by cPTIO, reaching levels comparable to those observed under water deficit conditions (Fig. 5A). This result indicates that NO contributes to the maintenance of membrane integrity under non-stress conditions by preventing lipid peroxidation even when water availability is not limiting. These results reinforce the dual role of NO. In addition to acting as a stress-induced signaling molecule, NO appears to function as a constitutive regulator of basal redox homeostasis. Thus, the removal of endogenous NO under adequate irrigation (control + cPTIO) led to increased lipid peroxidation, indicating that NO plays a constitutive role in fine-tuning redox balance and preserving membrane stability even under non-stress conditions.

Mata-Pérez et al. (2023) highlighted that NO acts as a key regulator of membrane stability by protecting lipids from oxidative damage, interacting with free radicals and limiting the propagation of lipid peroxidation, thereby preserving membrane integrity. In agreement, Khan et al. (2023) demonstrated that NO exerts a basal protective role against oxidative stress even under non-stress conditions. As normal cellular metabolism continuously generates reactive oxygen species, NO functions as an essential modulator of redox homeostasis, preventing the gradual accumulation of oxidative damage throughout plant development.

In contrast, under water deficit conditions, leaf MDA concentration was elevated regardless of cPTIO application, suggesting that drought-induced oxidative damage may override the protective effects of endogenous NO (Fig. 5A). However, exogenous NO supply has been reported as a factor that increases tolerance to oxidative stress, for example, exogenous NO application has been shown to mitigate oxidative stress and increase antioxidant defenses in perennial crops such as sugarcane. In sugarcane plants subjected to water deficit, foliar application of the NO donor, *S*-nitrosoglutathione (GSNO), increased antioxidant enzyme activities and prevented oxidative damage, leading to improved physiological performance under stress conditions (Silveira et al., 2017a). The reduction in root CAT activity following the removal of endogenous NO by cPTIO under both well-watered and water deficit conditions suggests that NO positively regulates CAT activity in roots, independently of plant water status (Fig. 6B). However, this modulation was not accompanied by changes in H_2O_2 accumulation (Fig. 5C, D), indicating that CAT downregulation alone was not sufficient to alter H_2O_2 levels.

There is evidence that some polyamines (PAs) can modulate NO biosynthesis, as the exogenous supply of spermidine and spermine induces a rapid increase in NO production in root tips and young leaves of Arabidopsis (Wimalasekera et al., 2011). In citrus plants, polyamines were shown to induce protein *S*-nitrosation, a key NO-mediated post-translational modification. *S*-nitrosation modulates protein activity, stability, and subcellular localization and is recognized as a central mechanism in redox regulation. In this context, Tanou et al. (2014) demonstrated the *S*-nitrosation of catalase, which was accompanied by enhanced enzymatic activity. Together, these findings support that PAs may influence redox homeostasis through NO-dependent mechanisms, reinforcing the role of NO as a fine-tuning regulator of antioxidant defenses.

5. Conclusion

In conclusion, our findings demonstrate that NO acts as an important integrator of hydraulic and redox processes in citrus. NO regulated aquaporins in an organ-specific manner, acting as a negative regulator of PIP2.1 in leaves under drought while promoting PIP1 expression in roots to support water uptake. In addition, NO exerted a dual effect on shoot hydration, under well-watered conditions, endogenous NO contributed to higher leaf water status, whereas under water deficit it was associated with reduced leaf hydration. Furthermore, NO contributed to membrane integrity by limiting lipid peroxidation even under non-stress conditions, reinforcing its role in maintaining basal redox balance. Together, these findings highlight that the action of NO is not restricted to stress responses but also participates in the constitutive homeostasis of citrus plants, coordinating water transport and oxidative stress (Fig. S1).

Supplementary material

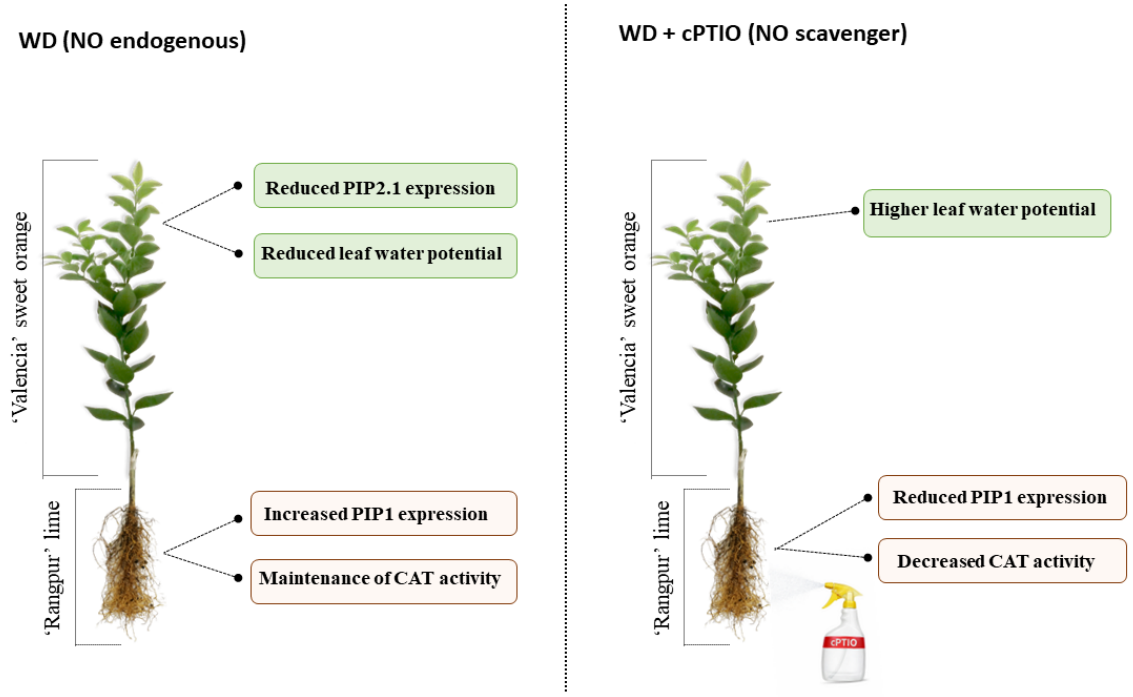

Figure S1. Schematic representation of the main responses in leaves and roots of 'Valencia' sweet orange scions grafted onto 'Rangpur' lime under water deficit (WD) and WD combined with cPTIO (WD + cPTIO).

References

Akaike, T., & Maeda, H. (1996). Quantitation of nitric oxide using 2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO). In: Methods in Enzymology. Academic Press, p. 211-221. https://doi.org/10.1016/S0076-6879(96)68023-9

Ayadi, M., Mieulet, D., Fabre, D., Verdeil, J. L., Vernet, A., Guiderdoni, E., & Masmoudi, K. (2014). Functional analysis of the durum wheat gene TdPIP2; 1 and its promoter region in response to abiotic stress in rice. *Plant Physiology and Biochemistry*, *79*, 98-108. https://doi.org/10.1016/j.plaphy.2014.02.018

Azevedo, R. A. de, Alas, R. M., Smith, R. J., & Lea, P. J. (1998). Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and azone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barbey. Physiologia Plantarum, 104(2), 280-292. doi:10.1034/j.1399-3054.1998.1040217.x

Boscariol-Camargo, R. L., Berger, I. J., Souza, A. A., Amaral, A. M. D., Carlos, E. F., Freitas-Astúa, J., Takita, M.A., Targon, M.L.P.N., Medina, C.L., Reis, M.S., Machado, M. A. (2007). In silico analysis of ESTs from roots of Rangpur lime (*Citrus limonia* Osbeck) under water stress. Genetics and Molecular Biology, 30: 906-916. https://doi.org/10.1590/S1415-47572007000500019

Bradford, M.N. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Bright, J., Desikan, R., Hancock, J. T., Weir, I. S., & Neill, S. J. (2006). ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H2O2 synthesis. The Plant Journal, v. 45, n. 1, p. 113–122, 2006. https://doi.org/10.1111/j.1365-313x.2005.02615.x

Cakmak, I., & Horst, W. J. (1991). Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (Glycine max). *Physiologia plantarum*, *83*(3), 463-468. https://doi.org/10.1111/j.1399-3054.1991.tb00121.x

Cimen, B., and Yesiloglu, T. (2016). Rootstock Breeding for Abiotic Stress Tolerance in *Citrus*. In Abiotic and Biotic Stress in Plants - Recent Advances and Future Perspectives (Issue February, pp. 527–563). InTech. https://doi.org/10.5772/62047

Corpas, F. J., & Barroso, J. B. (2017). Nitric oxide synthase-like activity in higher plants. Nitric oxide: biology and chemistry, *68*, 5-6. 10.1016/j.niox.2016.10.009

Desikan, R., Cheung, M. K., Bright, J., Henson, D., Hancock, J. T., & Neill, S. J. (2004). ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. Journal of experimental botany, 55: (395), 205-212. https://doi.org/10.1093/jxb/erh033

Di Pietro, M., Vialaret, J., Li, G. W., Hem, S., Prado, K., Rossignol, M., Maurel, C., & Santoni, V. (2013). Coordinated post- translational responses of aquaporins to abiotic and nutritional stimuli in Arabidopsis roots. Molecular & Cellular Proteomics, 12: 3886-3897.

Edwards, G. E., & Baker, N. R. (1993). Can CO2 assimilation in maize leaves be predicted accurately from chlorophyll fluorescence analysis?. *Photosynthesis research*, *37*(2), 89-102. https://doi.org/10.1007/BF02187468

Giannopolitis, C. N., & Ries, S. K. (1977). Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol. 59, 309–314. https://doi.org/10.1104/pp.59.2.309

Girardi, E. A., Brandão, A. D., Coelho, R. D., Couto, H. T. Z. D., Buckeridge, M. S., & Mourão Filho, F. D. A. A. (2018). Polyethylene glycol damages grafted citrus plants based on biometric, physiological, and biochemical responses. Revista Brasileira de Fruticultura, 40: 1-7, 2018. https://doi.org/10.1590/0100-29452018071

Hoagland, D.R; Arnon, D.I. (1950). The water-culture method for growing plants without soil. Berkeley: California Agricultural Experiment Station, Circular nº 347, 1950. 39p. https://doi.org/10.1111/ppl.12575

Hu, W., Yuan, Q., Wang, Y., Cai, R., Deng, X., Wang, J., ... & He, G. (2012). Overexpression of a wheat aquaporin gene, TaAQP8, enhances salt stress tolerance in transgenic Tobacco. Plant and Cell Physiology, *53*(12), 2127-2141. https://doi.org/10.1093/pcp/pcs154

Javot, H., & Maurel, C. (2002). The role of aquaporins in root water uptake. Annals of Botany, 90: 301-313, 2002. https://doi.org/10.1093/aob/mcf199

Jeandroz, S., Wipf, D., Stuehr, D. J., Lamattina, L., Melkonian, M., Tian, Z., Zhu, Y., Carpenter, J.E., Wong, K. G., & Wendehenne, D. (2016). Occurrence, structure, and evolution of nitric oxide synthase–like proteins in the plant kingdom. *Science Signaling*, *9*(417), re2-re2. 10.1126/scisignal.aad4403

Khan, M., Ali, S., Al Azzawi, T. N. I., & Yun, B. W. (2023). Nitric oxide acts as a key signaling molecule in plant development under stressful conditions. International Journal of Molecular Sciences, 24(5), 4782. https://doi.org/10.3390/ijms24054782

Kolbert, Z. S., Barroso, J. B., Brouquisse, R., Corpas, F. J., Gupta, K. J., Lindermayr, C., G.J. Loake, J.M. Palma, M. Petřivalský, D. Wendehenne, & Hancock, J. T. (2019). A forty year journey: The generation and roles of NO in plants. Nitric oxide, 93, 53-70. https://doi.org/10.1016/j.niox.2019.09.006

Kumari, A., Kaladhar, V. C., Yadav, N., Singh, P., Reddy, K., & Gupta, K. J. (2023). Nitric oxide regulates mitochondrial biogenesis in plants. Plant, cell & environment, 46(8), 2492-2506. https://doi.org/10.1111/pce.14637

Liu, H. Y., Yu, X., Cui, D. Y., Sun, M. H., Sun, W. N., Tang, Z. C., .Kwak, S.S., & Su, W. A. (2007). The role of water channel proteins and nitric oxide signaling in rice seed germination. Cell research, 17: 638-649, 2007. https://doi.org/10.1038/cr.2007.34

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25(4), 402-408. https://doi.org/10.1006/meth.2001.1262

Machado, R. S., Ribeiro, R. V., Marchiori, P. E. R., Machado, D. F. S. P., Machado, E. C., & Landell, M. G. D. A. (2009). Respostas biométricas e fisiológicas ao déficit hídrico em cana-de-açúcar em diferentes fases fenológicas. Pesquisa Agropecuária Brasileira. 2009; 44: 1575-1582. https://doi.org/10.1590/S0100-204X2009001200003

Mafra, V., Kubo, K. S., Alves-Ferreira, M., Ribeiro-Alves, M., Stuart, R. M., Boava, L. P., Rodrigues, C.M., & Machado, M. A. (2012). Reference genes for accurate transcript normalization in citrus genotypes under different experimental conditions. *PloS one*, *7*(2), e31263. https://doi.org/10.1371/journal.pone.0031263

Mata-Pérez, C., Sánchez-Vicente, I., Arteaga, N., Gómez-Jiménez, S., Fuentes-Terrón, A., Oulebsir, C. S., Calvo-Polanco, M., Oliver, C., & Lorenzo, Ó. (2023). Functions of nitric oxide-mediated post-translational modifications under abiotic stress. *Frontiers in Plant Science*, *14*, 1158184. https://doi.org/10.3389/fpls.2023.1158184

Maurel, C., Boursiac, Y., Luu, D. T., Santoni, V., Shahzad, Z., & Verdoucq, L. (2015). Aquaporins in plants. .Physiological Reviews, 95: 1321–1358. https://doi.org/10.1152/physrev.00008.2015

Medina, C. L., & Machado, E. C. (1998). Gas exchange and water relations of "Valencia" orange tree grafted on "Rangpur" lime and *Poncirus trifoliata*, submitted to a water deficit. Bragantia (Brazil), *57*(1):15-22.

Medina, C. L., Machado, E. C., & Pinto, J. M. (1998). Fotossíntese de laranjeira'Valência'enxertada sobre quatro porta-enxertos e submetida à deficiência hídrica. Bragantia, 57: 1-14. https://doi.org/10.1590/S0006-87051998000100001

Miranda, M. T., da Silva, S. F., Moura, B. B., Hayashi, A. H., Machado, E. C., & Ribeiro, R. V. (2018). Hydraulic redistribution in Citrus rootstocks under drought. Theoretical and Experimental Plant Physiology, 30: 165-172, 2018. https://doi.org/10.1007/s40626-018-0111-8

Miranda, M. T., Da Silva, S. F., Silveira, N. M., Pereira, L., Machado, E. C., & Ribeiro, R. V. (2021). Root osmotic adjustment and stomatal control of leaf gas exchange are dependent on citrus rootstocks under water deficit. Journal of Plant Growth Regulation, 40: 11-19, 2021. https://doi.org/10.1007/s00344-020-10069-5

Miranda, M. T., Espinoza-Núñez, E., Silva, S. F., Pereira, L., Hayashi, A. H., Boscariol-Camargo, R. L., Carvalho, S.A., Machado, E.C., Ribeiro, R. V. (2022). Water stress signaling and hydraulic traits in three congeneric citrus species under water deficit. Plant Science, 319: p. 111255, 2022. https://doi.org/10.1016/j.plantsci.2022.111255

Nakano, Y., & Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880. https://doi.org/10.1093/oxfordjournals.pcp.a076232

Pissolato, M. D., Silveira, N. M., Prataviera, P. J. C., Machado, E. C., Seabra, A. B., Pelegrino, M. T., Sodek, L., Ribeiro, R. V. (2020). Enhanced nitric oxide synthesis

through nitrate supply improves drought tolerance of sugarcane plants. Frontiers in Plant Science, 11: 970, 2020. https://doi.org/10.3389/fpls.2020.00970

Ribeiro, R.V., Espinoza-Núñez, E., Junior, J.P., Filho, F.A.A.M., Machado, E.C. (2014). Citrus Rootstocks for Improving the Horticultural Performance and Physiological Responses Under Constraining Environments. In: Ahmad, P., Wani, M., Azooz, M., Tran, LS. (eds) Improvement of Crops in the Era of Climatic Changes. Springer, New York, NY. https://doi.org/10.1007/978-1-4614-8830-9_1

Silveira, N. M., Frungillo, L., Marcos, F. C., Pelegrino, M. T., Miranda, M. T., Seabra, A. B., Salgado, I., Machado, E.C., Ribeiro, R. V. (2016). Exogenous nitric oxide improves sugarcane growth and photosynthesis under water deficit. Planta, 244: 181-190. https://doi.org/10.1007/s00425-016-2501-y.

Silveira, N.M., Marcos, F.C., Frungillo, L., Moura, B.B., Seabra, A.B., Salgado, I., Machado, E.C., Hancock, J.T., Ribeiro, R.V. (2017a). S-nitrosoglutathione spraying improves stomatal conductance, Rubisco activity and antioxidant defense in both leaves and roots of sugarcane plants under water deficit. Physiol. Plant. 160, 383–395. https://doi.org/10.1111/ppl.12575

Silveira, N. M., Hancock, J. T., Frungillo, L., Siasou, E., Marcos, F. C., Salgado, I., ... & Ribeiro, R. V. (2017b). Evidence towards the involvement of nitric oxide in drought tolerance of sugarcane. Plant physiology and biochemistry, v. 115, p. 354-359. https://doi.org/10.1016/j.plaphy.2017.04.011

Silveira, N. M., Prataviera, P. J., Pieretti, J. C., Seabra, A. B., Almeida, R. L., Machado, E. C., & Ribeiro, R. V. (2021a). Chitosan-encapsulated nitric oxide donors enhance physiological recovery of sugarcane plants after water deficit. Environmental and Experimental Botany, v. 190, p. 104593. https://doi.org/10.1016/j.envexpbot.2021.104593

Silveira, N. M., Ribeiro, R. V., Morais, S. F. N., Souza, S. C. R., Silva, S. F., Seabra, A. B., Hancock, J. T., Machado, E. C. (2021b). Leaf arginine spraying improves leaf gas exchange under water deficit and root antioxidant responses during the recovery period. Plant Physiology and Biochemistry, 162, 315-326. https://doi.org/10.1016/j.plaphy.2021.02.036

Silveira, N. M., de Oliveira, M. I. M., da Silva, S. F., Miranda, M. T., Passos, M. P., Machado, E. C., & Ribeiro, R. V. (2024). Enhanced root growth and nitric oxide content in 'Mandarin'lime under drought stress: experimental evidence. Brazilian Journal of Botany, 47(1), 13-18. https://doi.org/10.1007/s40415-023-00964-y

Tanou, G., Ziogas, V., Belghazi, M., Christou, A., Filippou, P., Job, D., ... & Molassiotis, A. (2014). Polyamines reprogram oxidative and nitrosative status and the proteome of citrus plants exposed to salinity stress. Plant, cell & environment, 37(4), 864-885. **https://doi.org/10.1111/pce.12204**

Velikova, V., Yordanov, I., & Edreva, A. J. P. S. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous

polyamines. Plant science, 151(1): 59-66. https://doi.org/10.1016/S0168-9452(99)00197-1

Wimalasekera, R., Tebartz, F., Scherer, G.F., 2011. Polyamines, polyamine oxidases and nitric oxide in development, abiotic and biotic stresses. Plant Sci. 181, 593–603. https://doi.org/10.1016/j.plantsci.2011.04.00

Zheng, Y., Xiao, J., Zheng, K., Ma, J., He, M., Li, J., & Li, M. (2021). Transcriptome profiling reveals the effects of nitric oxide on the growth and physiological characteristics of watermelon under aluminum stress. *Genes*, *12*(11), 1735. https://doi.org/10.3390/genes12111735

CONSIDERAÇÕES FINAIS

O déficit hídrico afeta negativamente a produtividade da citricultura, especialmente em um cenário de crescente variabilidade climática. Compreender os mecanismos fisiológicos e moleculares que sustentam a manutenção do status hídrico e da integridade estrutural das plantas é essencial para avançar no entendimento da tolerância à seca em espécies perenes.

Os resultados deste estudo demonstram que o NO atua como um importante integrador dos processos hidráulicos e redox em citros, modulando a expressão de aquaporinas de maneira órgão-específica e influenciando o estado hídrico da parte aérea. Além disso, o NO contribuiu para a manutenção da integridade de membranas e do equilíbrio redox basal, evidenciando que sua atuação não se restringe às condições de estresse, mas também participa da homeostase constitutiva da planta.

Entretanto, investigar a sinalização por NO envolve desafios consideráveis. Trata-se de uma molécula altamente reativa, de curta meia-vida, capaz de interagir com múltiplos alvos celulares, incluindo espécies reativas de oxigênio, hormônios como o ABA e outros sinalizadores. Essa natureza dinâmica e contexto-dependente pode resultar em respostas contrastantes, como observado neste estudo, no qual o efeito do NO sobre o status hídrico variou de acordo com o regime de irrigação. Dessa forma, este estudo contribui para uma compreensão mais integrada da coordenação entre transporte de água e sinalização redox em citros, reforçando a importância de abordagens fisiológicas e moleculares integrativas para elucidar a complexidade da sinalização vegetal.

APÊNDICE

Figura 1. Visão geral do experimento. Plantas de laranja Valência enxertadas em limoeiro ‘Cravo’, em condições de boa hidratação (controle), controle + cPTIO, déficit hídrico (DH) e DH + cPTIO.

Figura 2. Adição de PEG à solução nutritiva para indução do déficit hídrico (em A) e aplicação do sequestrador de NO (cPTIO) nas raízes do porta-enxerto limoeiro ‘Cravo’ em plantas de laranja ‘Valência’ (em B).