Multi-Environment Transcriptomic Analysis of Alfalfa Root Responses to Sunflower Intercropping

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Alfalfa (*Medicago sativa* L.) is a perennial forage legume valued for its high yield, forage nutritive value, and role in sustainable cropping systems. Because alfalfa typically produces low forage yield during its establishment year, companion cropping with annual species can generate economic returns during this phase. However, the impact that neighboring crops have on the molecular mechanisms of alfalfa roots under field conditions remains poorly understood. This study analyzed the transcriptomic responses of alfalfa roots grown in monoculture or intercropped with sunflower (*Helianthus annuus* L.) across multiple field environments. Root samples were collected from experiments in Hickson, ND, in 2021 and 2022; Prosper, ND, in 2021; and Red Lake Falls, MN, in 2022. Samples were collected at two developmental stages, late vegetative (TP 1) and early bud (TP 3), and RNA-seq was used to analyze treatment- and environment-associated changes in gene expression. A total of 49,095 *Medicago sativa* genes were quantified from the RNAseq data, with 45,686 genes showing detectable expression. After filtering out genes that did not meet the minimum expression threshold of 10 counts in at least 3 samples, 31,028 genes remained for differential expression analysis. Furthermore, among the 49,095 *Medicago sativa* genes, 33,296 had identifiable *Arabidopsis thaliana* orthologs, which were used for functional annotation and gene ontology enrichment analysis. Intercropping triggered significant changes in gene expression, most of which were environment-specific, although some differentially expressed genes were conserved across all locations. Gene ontology analysis of conserved genes revealed enrichment for hypoxia, anoxia, and other stress-related signaling pathways at TP1. Additionally, genes associated with ethylene signaling and reactive oxygen species were enriched at TP1, consistent with an early stress-signaling response in alfalfa roots exposed to sunflower roots. By TP3, conserved genes were enriched for processes related to cell wall organization and phenylpropanoid biosynthesis, indicating later-stage structural and metabolic adjustment in intercropped alfalfa roots. Overall, these findings support a two-phase transcriptional response in alfalfa roots to sunflower intercropping, beginning with the detection and signaling of initial stress, followed by structural reinforcement. These results on molecular mechanisms associated with belowground plant-plant interactions provide insights into developing productive, resilient, and sustainable intercropping systems.

Keywords: Alfalfa; intercropping; RNA-seq; plant–plant interactions

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