

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

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Why does alfalfa matter?

One of the most important perennial forage crops



- High-quality, protein-rich feed for livestock
- Fixes atmospheric nitrogen
- Improves soil health and supports sustainable cropping systems

The problem

Alfalfa has one major limitation



- During the establishment year, productivity is often low
- Growers may not see high returns right away
- Makes adoption more difficult, even though the crop has clear long-term value

Could intercropping help?

Intercropping may improve establishment-year system productivity

- Sunflower may provide grain or forage yield while alfalfa is still establishing
- Differences in crop growth and canopy structure may improve the use of light, water, nutrients, and land
- Wider sunflower rows may reduce shading and competition, supporting alfalfa establishment

What happens belowground?

The biological effects on alfalfa roots remain unclear

- Sunflower may alter the belowground environment experienced by alfalfa roots
- Root competition may affect water and nutrient availability
- Intercropping may also modify signaling, defense, and stress-response pathways in alfalfa
- These responses are not well understood under field conditions.

Objective and hypotheses

A field-based transcriptomic test of how alfalfa roots respond to sunflower intercropping

Objective

Determine whether sunflower intercropping alters alfalfa root gene expression across environments, row-spacing treatments, and growth stages

Hypothesis 1

Sunflower presence drives a stronger alfalfa root response than row spacing alone

Hypothesis 2

Responses are repeatable across field environments and changes over time

Field design: environments, treatments, and stages

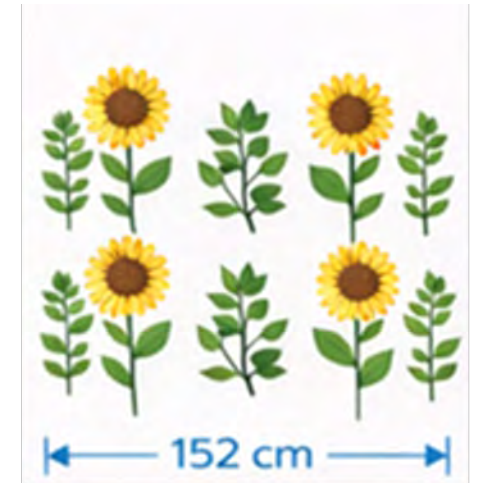
Root samples came from alfalfa grown alone or with sunflower under field conditions



Alfalfa
monocrop
(AL)



Alfalfa intercropped
76-cm row spacing
(ALIC76)



Alfalfa intercropped
152-cm row spacing
(ALIC152)

4 environments

Hickson 2021/2022
Prosper 2021
Red Lake Falls 2022

3 alfalfa systems

AL, ALIC76, ALIC152

2 sampling stages

TP1: early establishment
TP3: early bud

From field roots to differential gene expression

The analysis focused on alfalfa root transcriptomic responses under intercropping



- Root tissue collected from field-grown alfalfa
- RNA extracted and sequenced using poly(A)-selected RNA-seq libraries
- Reads aligned to the *Medicago sativa* Zhongmu No. 1 reference genome
- Annotation file rebuilt from GFF3 to GTF for feature counting
- Differential expression tested using DESeq2 in R

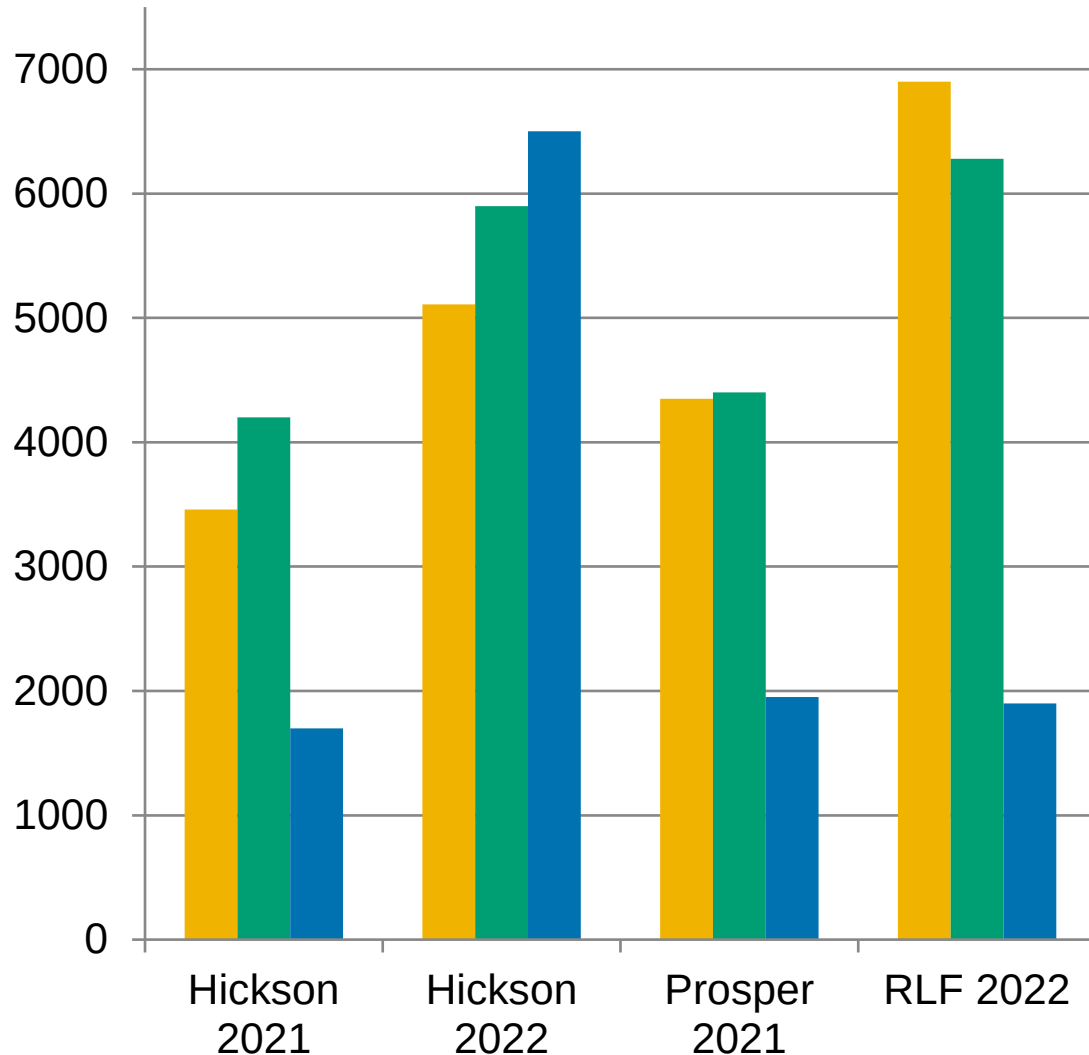
Key software

HISAT2 v2.2.1
featureCounts v2.0.4
R v4.4.3
DESeq2 v1.46.0
apeglm v1.28.0

Main biological contrast: alfalfa monocrop vs alfalfa intercropped with sunflower.

Result 1: sunflower presence drove the strongest root response

Across environments, contrasts involving sunflower produced thousands of DEGs



Key interpretation

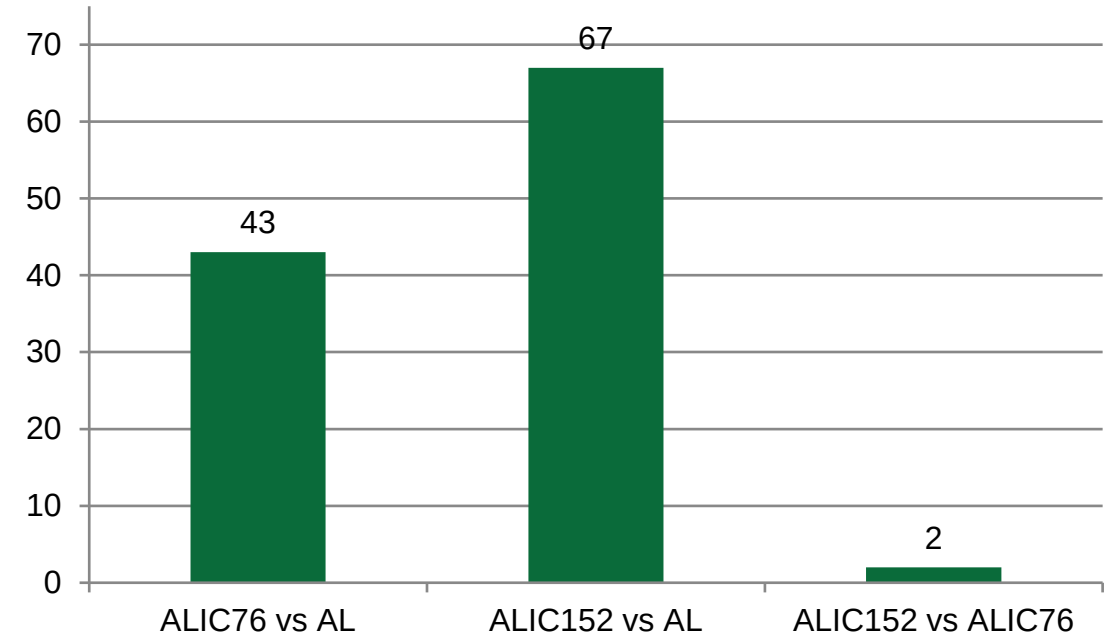
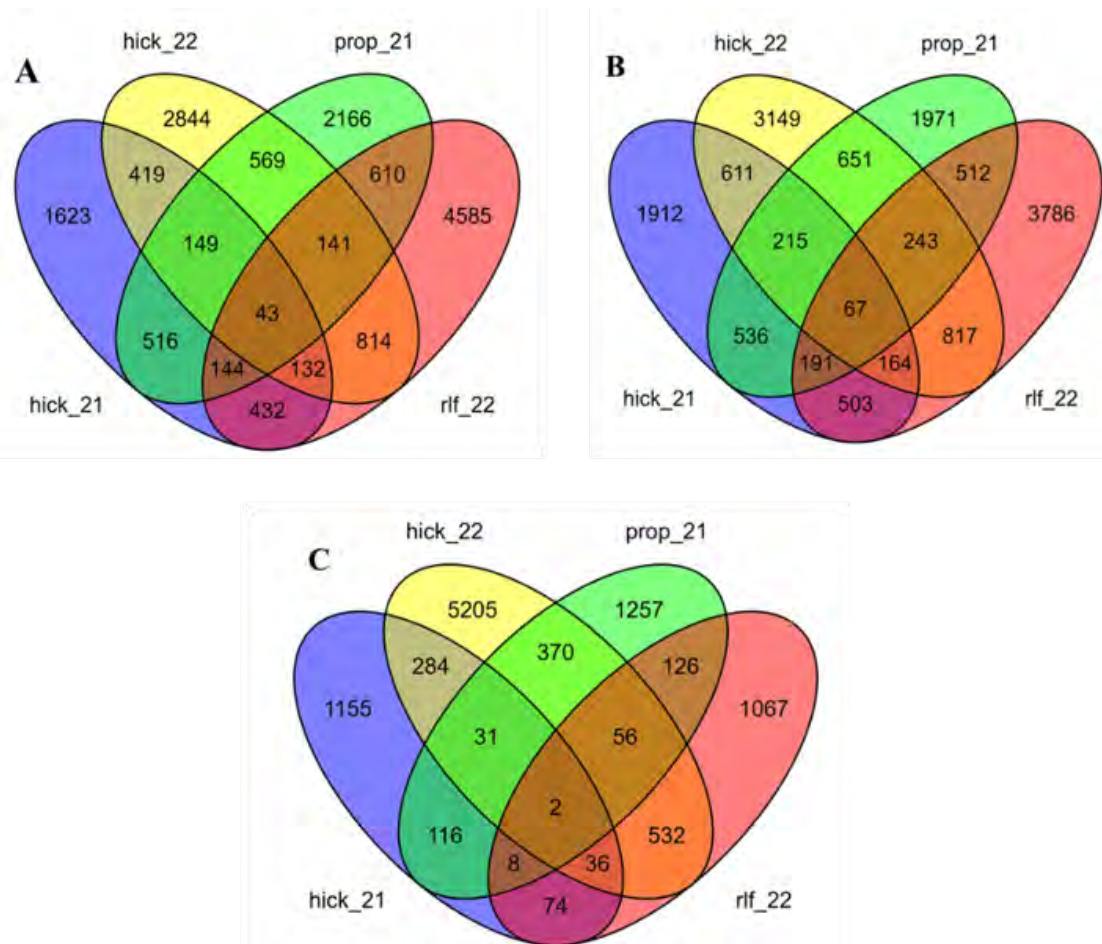
The presence of sunflower changed alfalfa root gene expression more consistently than spacing alone

Main message: what alfalfa grows with matters, not only how rows are spaced

DEGs = differentially expressed genes

Result 2: a shared core response was detected across fields

Most responses were environment-specific, but some genes were shared across all four environments at TP1



Repeatability matters because it separates a consistent biological signal from local environmental noise

Result 3: the root response changed over time

Alfalfa showed an early sensing/stress response followed by later structural and defense-related adjustment

Early establishment: TP1

- Oxygen-related signaling
- Hypoxia/stress response
- Rapid adjustment to changed soil conditions

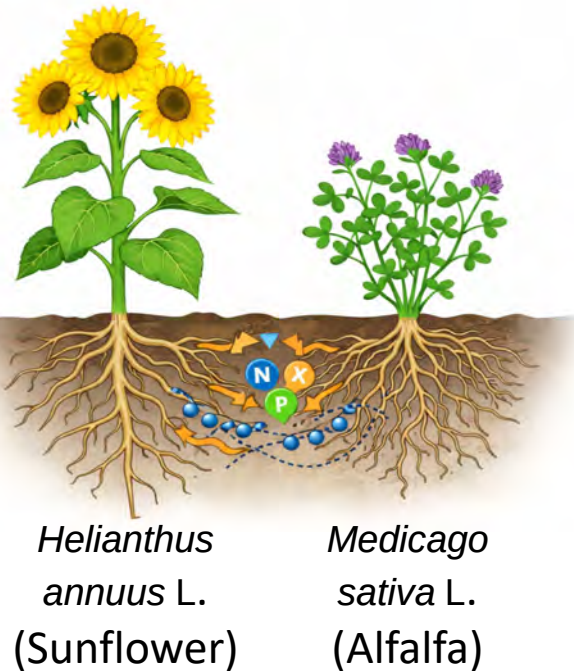
Later stage: TP3

- Defense
- Phenylpropanoid metabolism
- Lignin and cell-wall remodeling

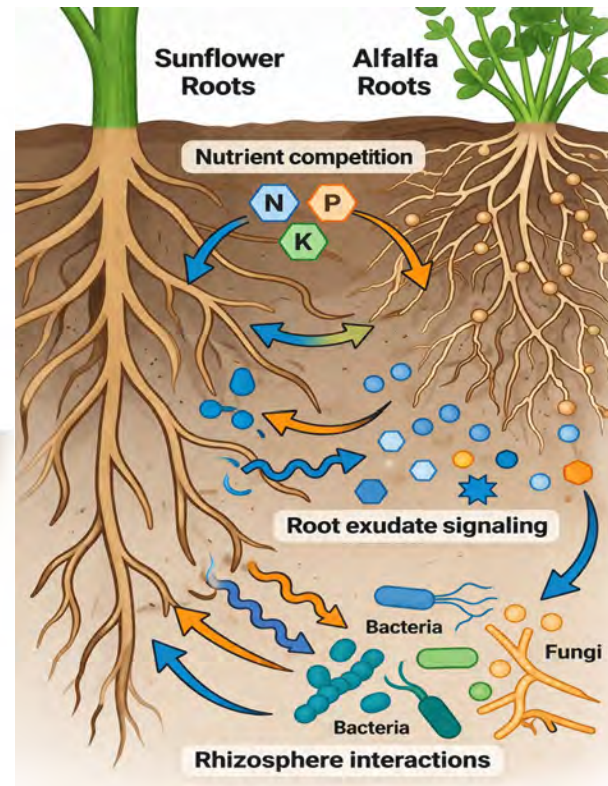
Interpretation: alfalfa roots first sense the changed belowground environment, then adjust root function

Working model: sunflower changes the alfalfa root environment

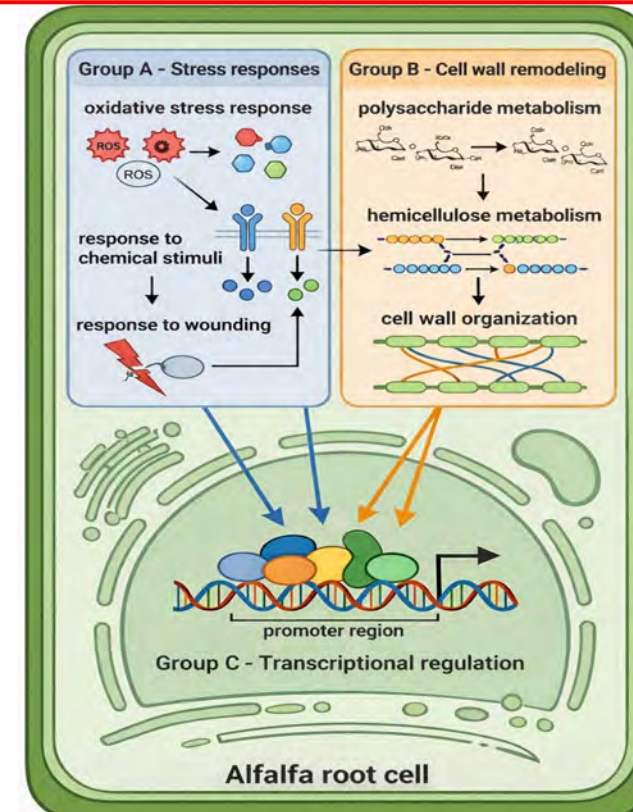
Shared field environment



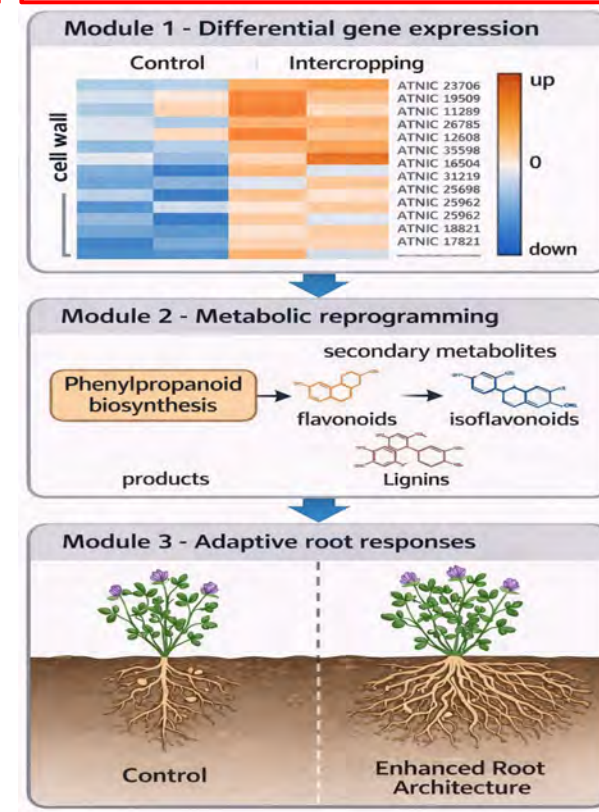
Root interactions belowground



Molecular response in alfalfa root cell



Functional outcomes



This is not passive competition. It is an active biological response to a neighboring crop

Implications for alfalfa improvement and intercropping

Transcriptomics identifies candidate processes that may support establishment under companion cropping

What does this study contribute?

- Field evidence that sunflower intercropping alters alfalfa root gene expression
- Recurrent pathways that may guide screening for intercropping-compatible germplasm
- A basis for linking molecular response with establishment, persistence, and system productivity

Interpretation limits and next steps

- Root transcriptomics alone cannot distinguish competition, signaling, facilitation, or microbial mediation
- Future work should integrate nodulation, nitrogen fixation, root traits, exudates, and microbiomes

Take-home message

Sunflower intercropping triggers a dynamic and repeatable alfalfa root transcriptomic response

1. Sunflower intercropping modifies the alfalfa root-zone environment
2. Alfalfa actively reprograms gene expression rather than simply being suppressed
3. The response is environment-sensitive, but a shared core signal is detectable

Intercropping is not just a practice; it is a biological system we can optimize

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Thank you

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