Microbial synthetic community ARC prevents aflatoxin and increases rhizobia-legume nodulation couplingly

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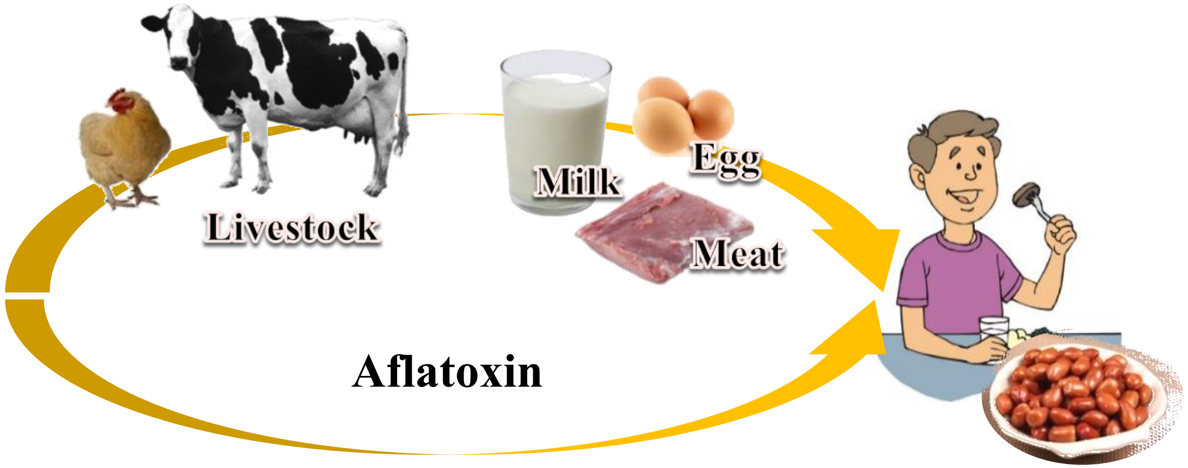
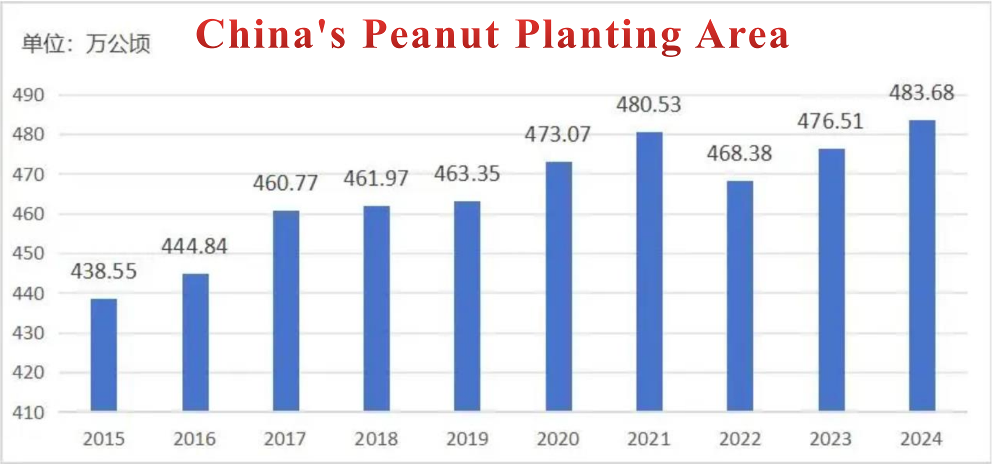
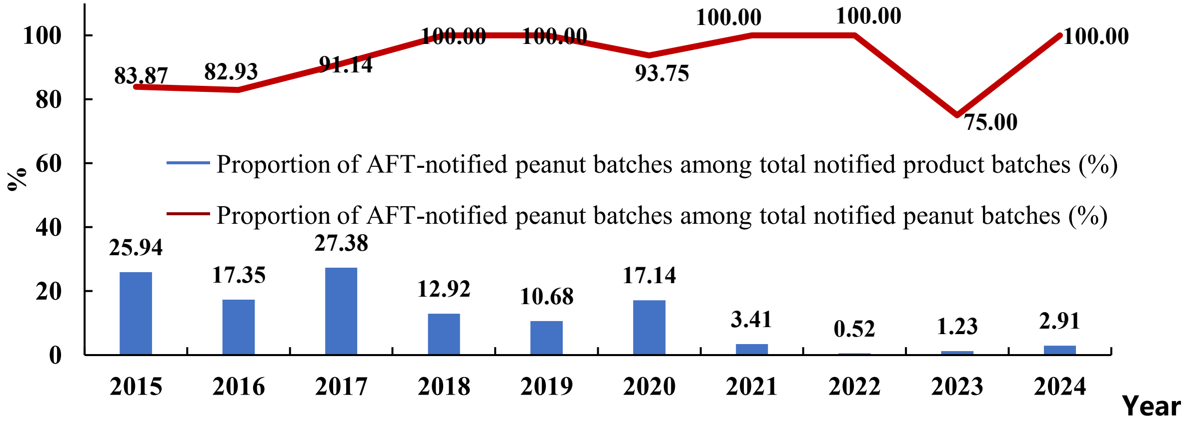
Background: Legume crops face two common global challenges in improving quality, yield, and safety

- Peanut is a wordwide crop for food, oil & feed. Boosting production capacity is a national strategic priority
- **Challenge 1 – Aflatoxin contamination**







Poor nodulation in peanut fields. Nodulation, N_2 fixation, fertilizer reduction, and yield increase: an unsolved challenge for over a century

- **Challenge 2 – Nodulation, nitrogen fixation, yield increase with reduced fertilizer**
- Symbiotic nitrogen fixation in leguminous plants is an important direction for green and sustainable agricultural development.
- There is always few nodules in field legumes

Peanut nodulation at pod-filling stage in Henan

1910, German rhizobial product

- **1886 –** Hermann reports pea nodule nitrogen fixation
- **1895 –** World’s first commercial rhizobial product
- Over 200 genes reported in legume-rhizobia symbiosis
- **AON theory**: (Peter Gresshoff, 1986)

  Excessive nodules weaken plant growth and yield

The main source of aflatoxin contamination in peanuts is identified as soil-borne *Aspergillus*

- A 20-year systematic monitoring of aflatoxin occurrence and distribution in Chinese peanut regions was conducted
- Fifty-three factors and their spatiotemporal links to aflatoxin contamination were analyzed – **soil-borne *Aspergillus* is identified as the primary source**

Contribution order	2009	2010	2011	2012	2013	2014	2015
1	T_{min}	AT_{10}	T_{min}	T_{max}	AT_{10}	RH_{min}	AT_{10}
2	AT_{10}	T_{mean}	AT_{10}	AT_{10}	T_{max}	T_{max}	T_{mean}
3	RH_{min}	R	T_{mean}	R	T_{min}	T_{min}	T_{max}
4	T_{mean}	RH_{min}	R	T_{mean}	R	AT_{10}	R
5	T_{max}	T_{min}	S	RH_{min}	SA	R	SA
6	R	T_{max}	RH_{min}	S	T_{mean}	T_{mean}	RH_{min}
7	S	S	T_{max}	RH_{mean}	RH_{mean}	RH_{mean}	T_{min}
8	AT_{20}	RH_{mean}	RH_{mean}	SA	S	S	S
9	SA	AT_{20}	SA	AT_{20}	RH_{min}	SA	AT_{20}
10	RH_{mean}	SA	AT_{20}	T_{min}	AT_{20}	AT_{20}	RH_{mean}

A "seesaw" effect was observed between aflatoxigenic fungi and rhizobia in the peanut rhizosphere

- By rhizosphere microbiome analysis, the abundances of *Aspergillus* and rhizobia in soil were shown to exhibit opposite trends – **a seesaw phenomenon**

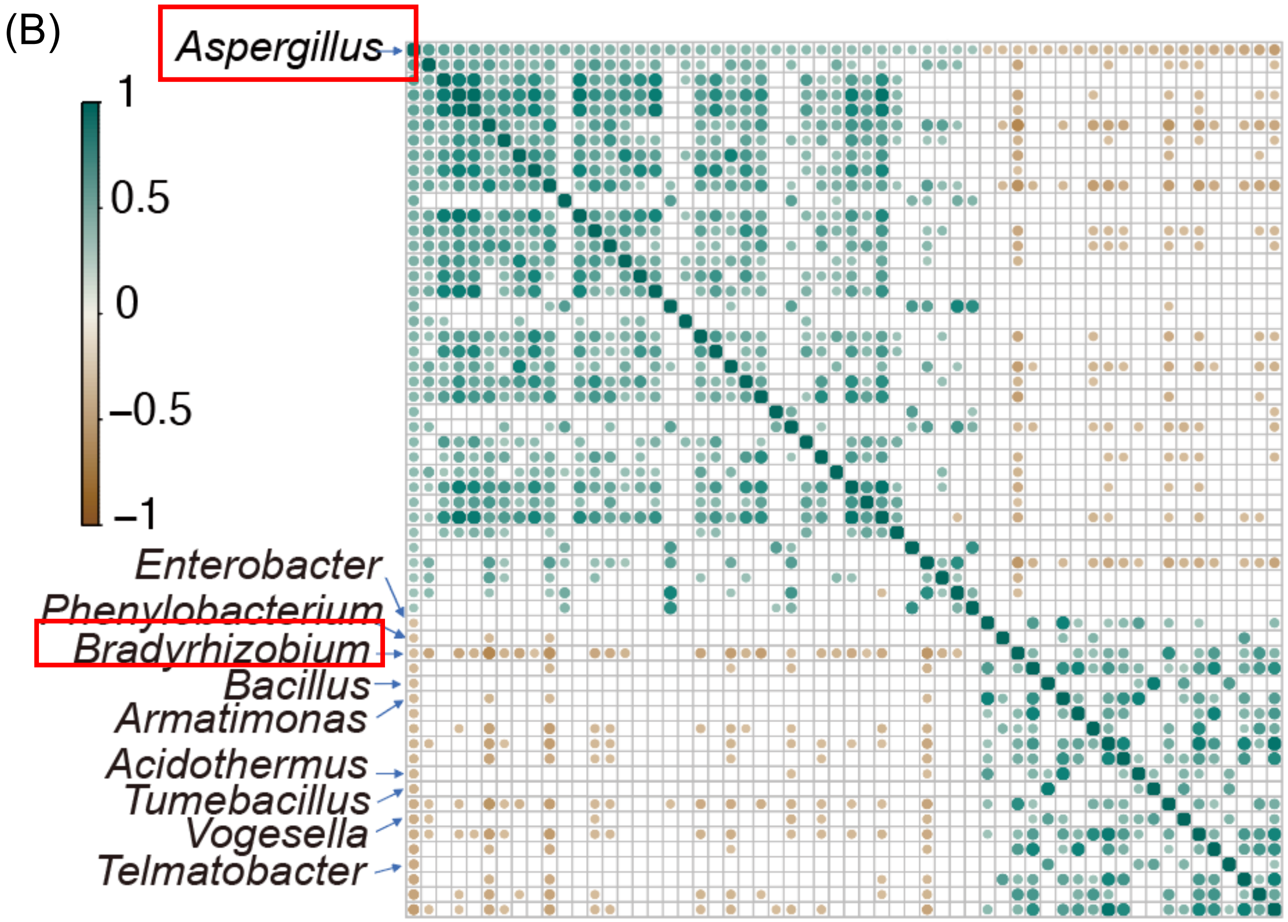
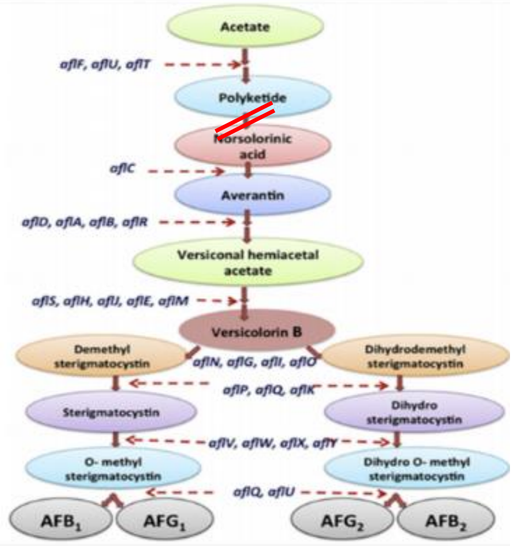


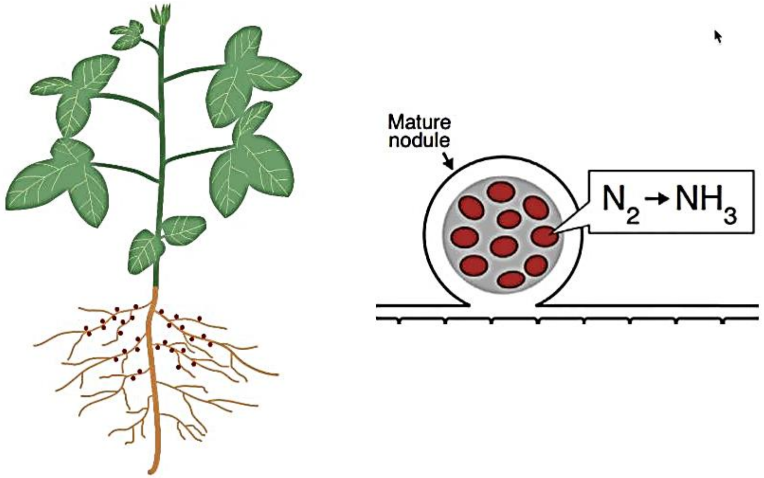
Figure 1(B) Correlations between rhizosphere microbial genera and *Aspergillus* abundance across regions.

Research overview – a scientific concept for coupling research was proposed

- A scientific concept was developed for a single technology to solve both problems: **biological coupling of soil-source aflatoxin control and promotion of nodulation-N_2 fixation**



Upstream blocking of the aflatoxin biosynthesis pathway



Peanut nodulation and nitrogen fixation

Microbiome structure deciphered, coupling bacteria screened

- 20 genera negatively correlated with *Aspergillus*, among which 8 genera with no negative correlation to rhizobia—**candidate coupling genera**

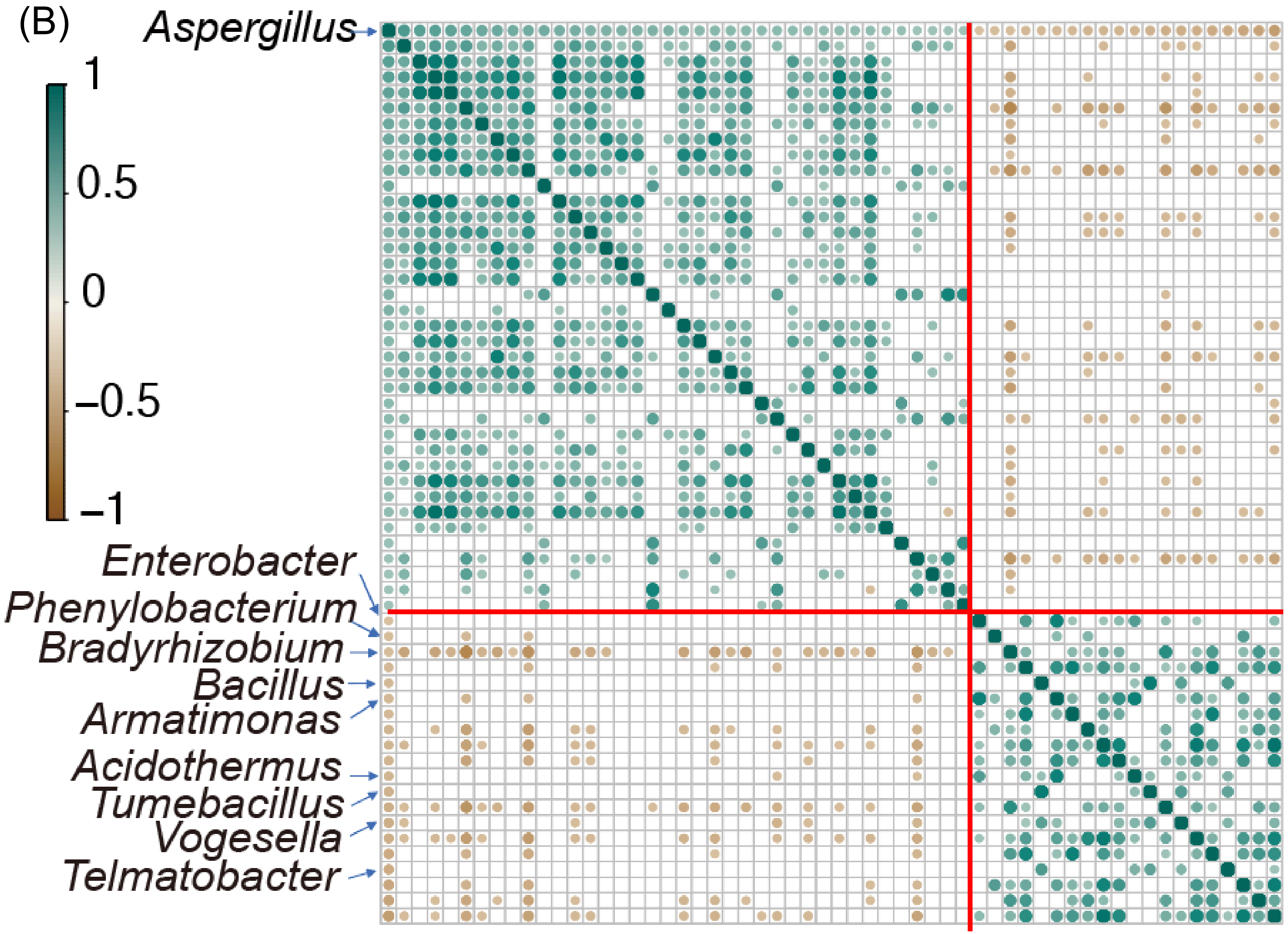


Figure 1(B) Correlations between rhizosphere microbial genera and *Aspergillus* abundance across regions.

Isolation, identification, strain establishment, functional characterization, and stepwise screening yielded four representative strains

- From the 8 genera, **127 strains were isolated** using culturomics technology
- In liquid co-culture and plate confrontation assays with *A. flavus*, 6 *Bacillus* strains and 1 *Enterobacter* strain were found to inhibit *A. flavus* mycelial growth, while 3 strains were found to inhibit rhizobia growth
- Based on **antifungal activity and rhizobial compatibility**, 3 representative *Bacillus* strains and 1 *Enterobacter* strain were selected for subsequent synthetic community assembly

(S1)

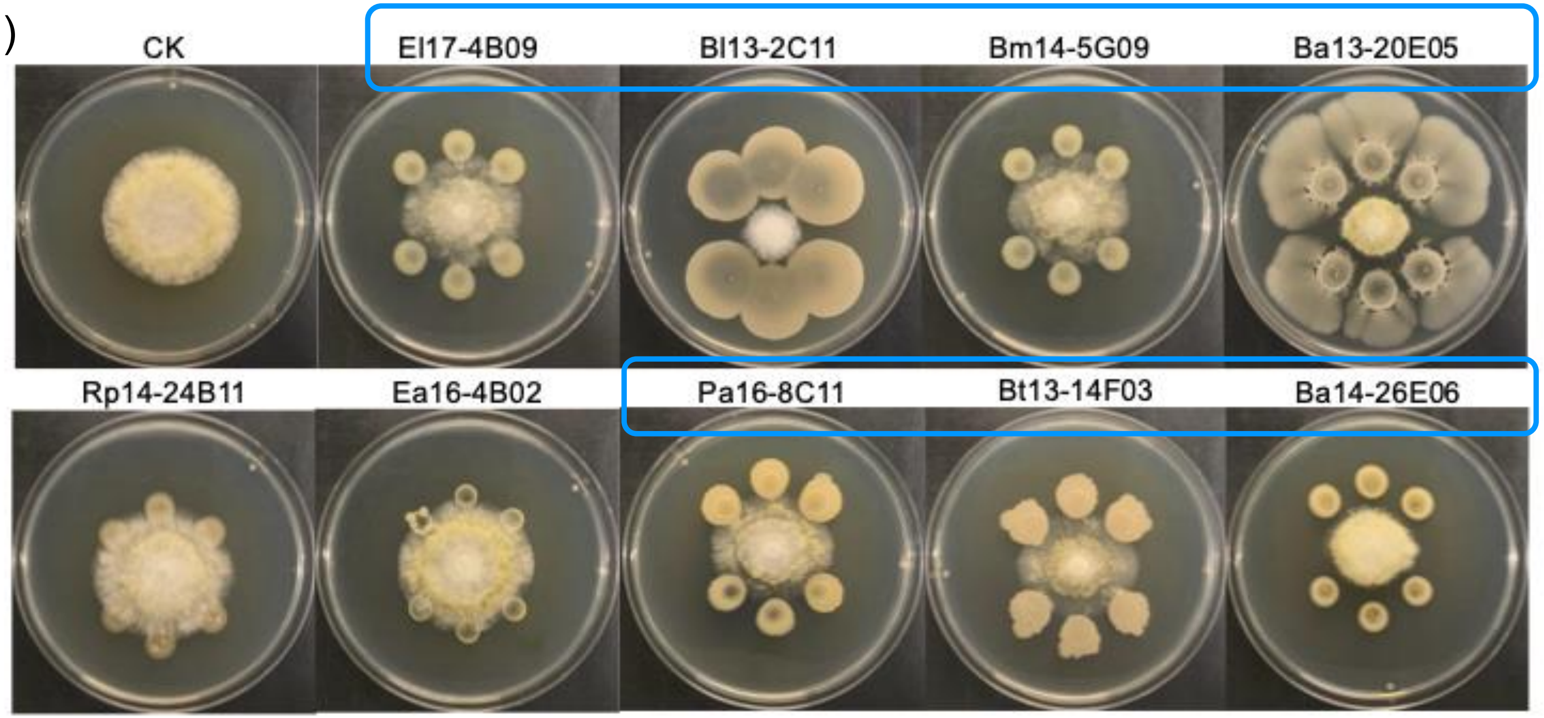


Figure S1 Plate confrontation assay between 9 candidate strains and *A. flavus*.

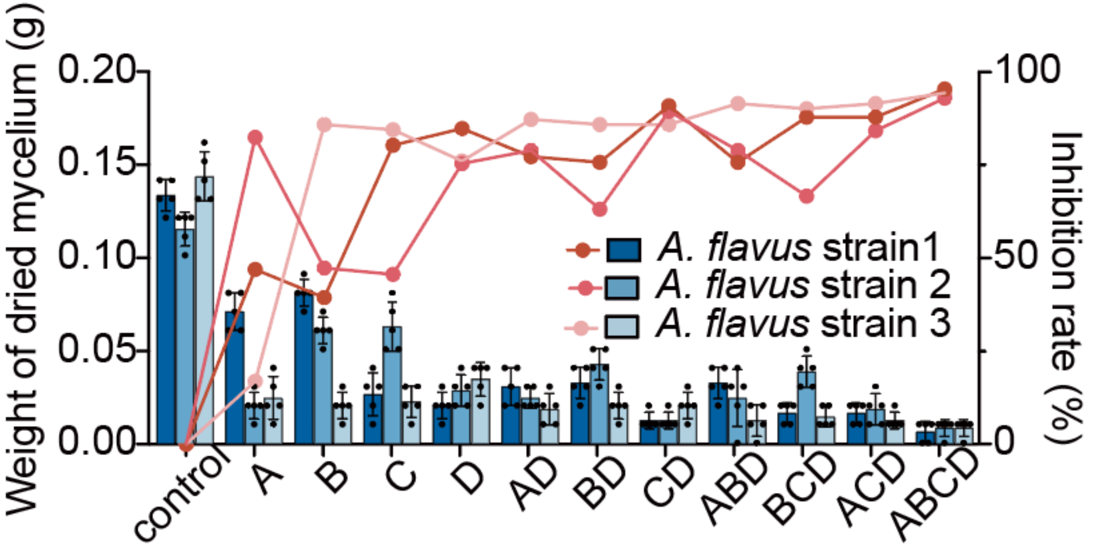
(S2)

Figure S2 Co-culture of 9 candidate strains and *Bradyrhizobium*.

Functional characterization identified the combination of 3 *Bacillus* + 1 *Enterobacter ludwigii* as the best

- Multiple SynComs were constructed and characterized using the 4 strains
- The **4-strain SynCom exhibited the best inhibition of *A. flavus*** versus single strains and other combinations
- This combination induced the **highest peanut nodule number** in field trials

(C)



(D)

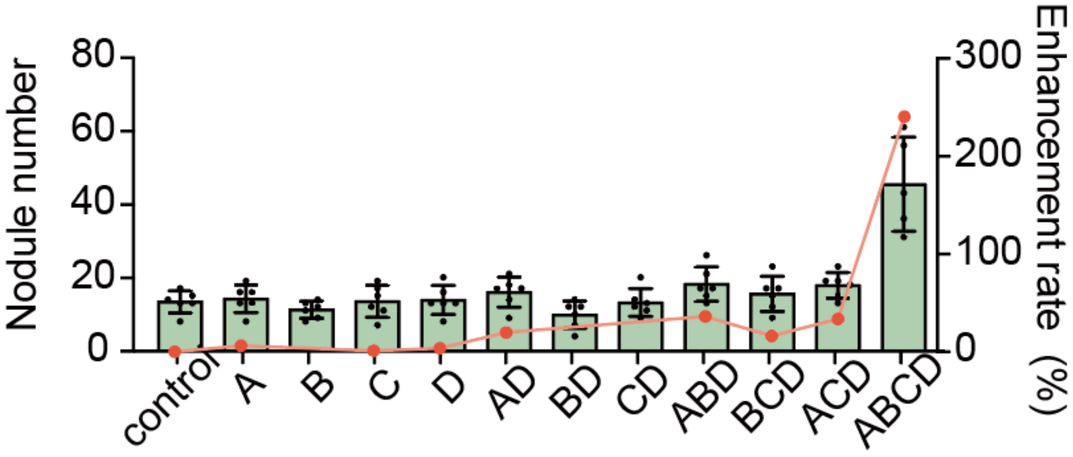


Figure 1(C) Inhibition of *A. flavus* by different combinations of strains within ARC. (D) Promoting the nodule number by different strain combinations within ARC.

Through systematic inoculation characterization, the ARC microbial agent was obtained, achieving coupled aflatoxin control and N_2 fixation

- **The 4-strain SynCom was named ARC**
- Gnotobiotic validation: **ARC alone induced no nodules**; co-inoculation with rhizobia increased nodule number and inhibited *Aspergillus*

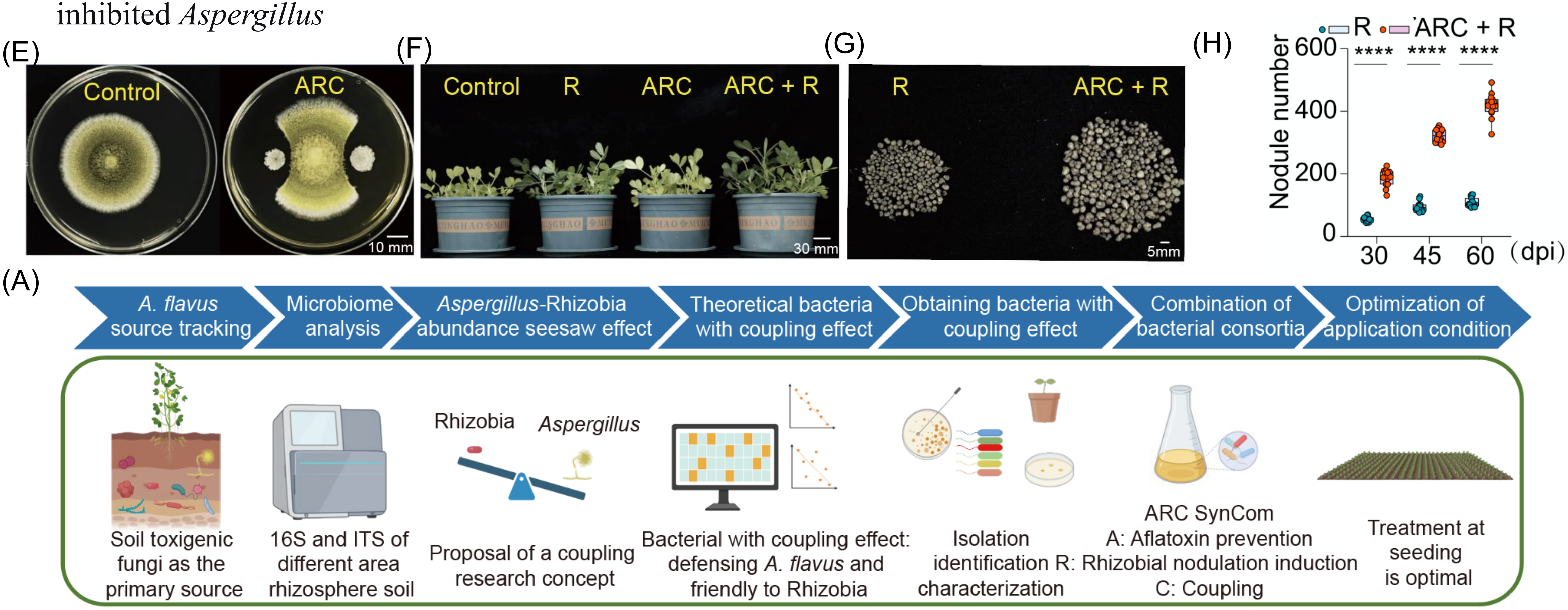
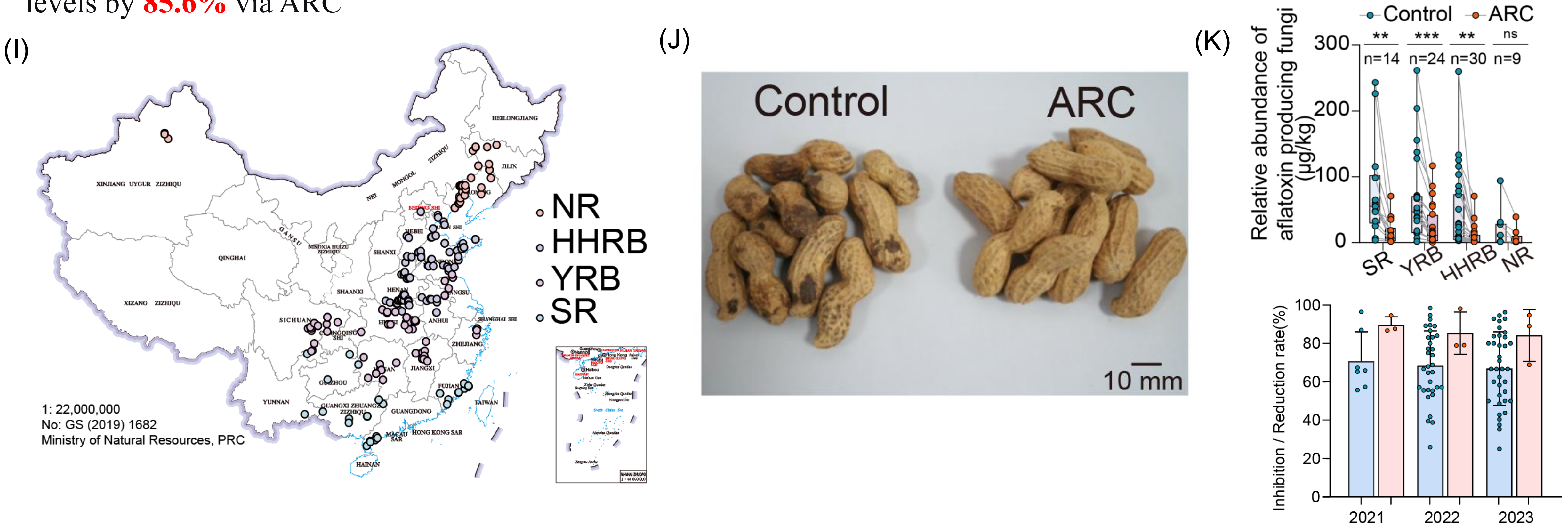


Figure1 (A) Workflow for developing ARC. (E) Colony morphology of *A. flavus* under control and ARC treatment in vitro. (F) Peanut plants phenotypes at 60 dpi under different pot treatment. (G) Nodule phenotypes at 60 dpi. (H) Nodule number measured.

Multi-year, multi-site field trial and demonstration results revealed that the ARC efficiently controls aflatoxin in peanuts

- A technical protocol and application model for ARC were established for large-scale field application. **Over 4 years, 325 sites across 19 provinces** were used for production trials and demonstrations
- Surface mold spots on peanuts were significantly reduced. Aflatoxigenic fungi were decreased by **63.2%–71.8%,** and aflatoxin levels by **85.6%** via ARC



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Ministry of Natural Resources, PRC

Figure 1(I) Distribution of national field trial sites. (J) Phenotype: Control versus ARC. (K) Abundances of aflatoxigenic fungi in peanuts from four major producing regions.

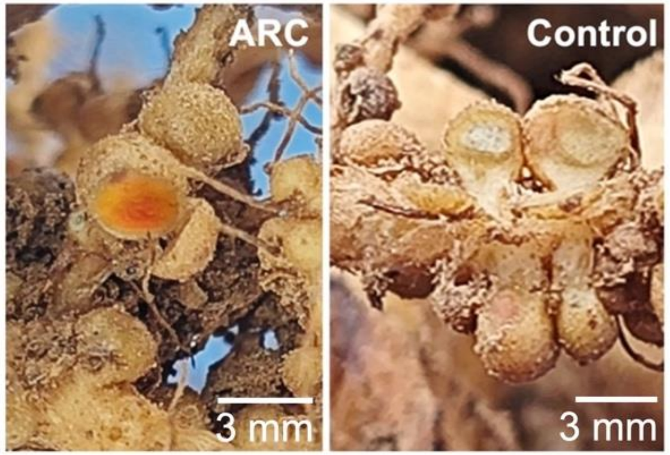
odulation and N_2 fixation were promoted by ARC across Chinese peanut regions

- Based on 325 field sites, average nodule number was increased **5.3-fold**, nitrogenase activity was increased **7.4-fold,** by ARC
- ARC-treated plants were found to **retain abundant active nodules at harvest**, thereby achieving continuous nitrogen supply throughout the growth period

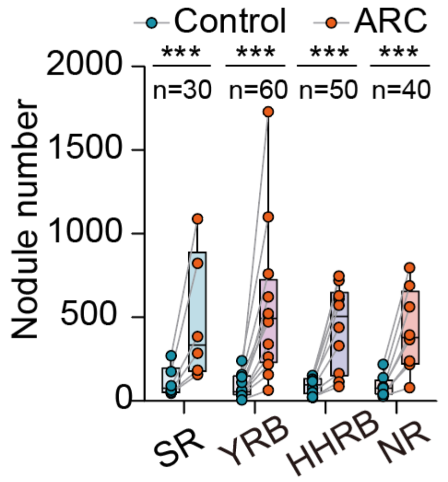
(L)

(M)

(S5)



(N)



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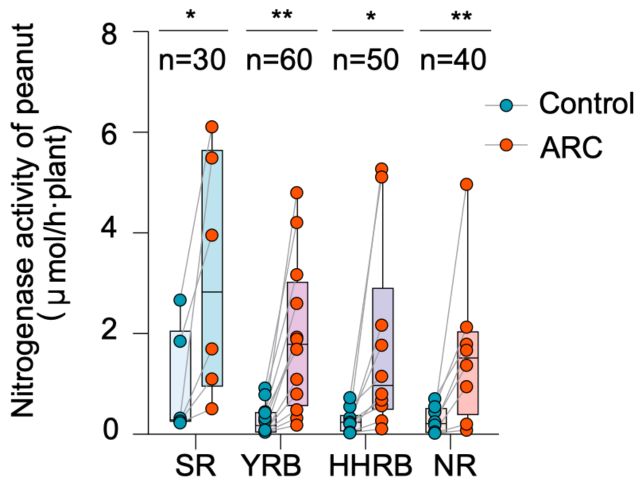
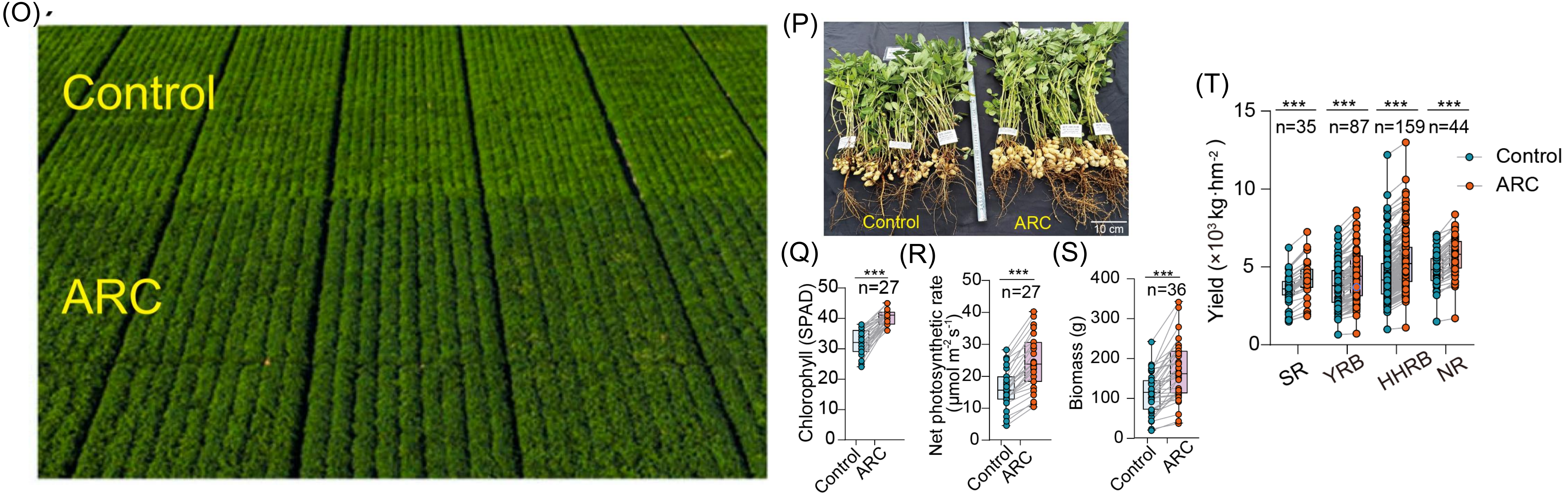


Figure1 (L, M) Close-up field comparison of roots without versus with ARC inoculant. (N) Nodule number across four major regions
Figure S4 The ARC applications in the fields increased the nitrogenase activity. Figure S5 Cross-sectional phenotype of peanut nodules at harvest.

19.8% yield increase achieved nationwide by ARC

- From 325 field trials, photosynthesis, vigorous growth, biomass, and yield traits were significantly enhanced by ARC
- **A 19.8% average yield increase** in multi-year, multi-site trials was confirmed by third-party organizations



First simultaneous aflatoxin control, super-nodulation, high N_2 fixation, and yield increase achieved by ARC in large-scale production

Figure1 (O) Aerial view of field plots without versus with ARC. (P) Field comparison of plant canopies. Statistical analysis of chlorophyll II (Q), net photosynthetic rate (R), and biomass (S). (T) Peanut yield across four regions.

ARC reshaped the structure of the peanut rhizosphere and root microbiome

- ARC significantly **enriched nitrogen-fixing bacteria** and **reduced pathogenic fungi**
- ARC increased *Bradyrhizobium* abundance and decreased *Aspergillus* abundance in rhizosphere soil and roots

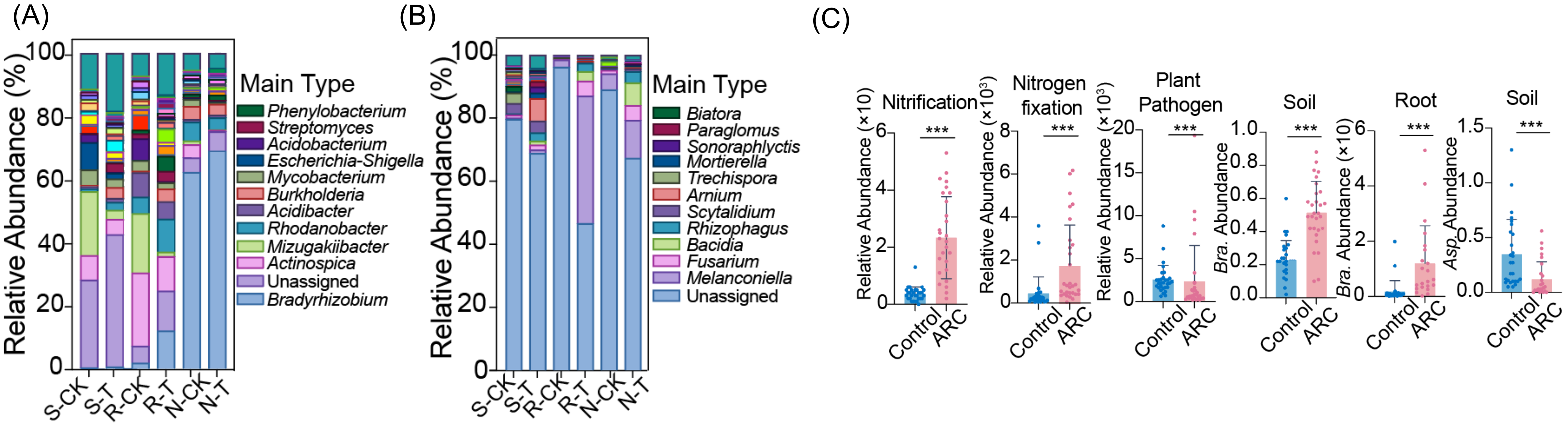


Figure 2 (A) Bacterial (left) and (B) fungal (right) community composition in rhizosphere soil, roots, and nodules after ARC application. (C) Abundances of nitrification, nitrogen-fixing, and plant-pathogenic bacteria (ARC/Control) and Bradyrhizobium in soil, roots, and nodules.

ARC damaged multiple biological processes in *A. flavus*

- Transcriptome analysis revealed that ARC downregulated genes enriched in DNA metabolism/repair, proteolysis, and fatty acid biosynthesis in *A. flavus*
- ARC compromised genome stability, proteostasis, and lipid metabolism in *A. flavus*

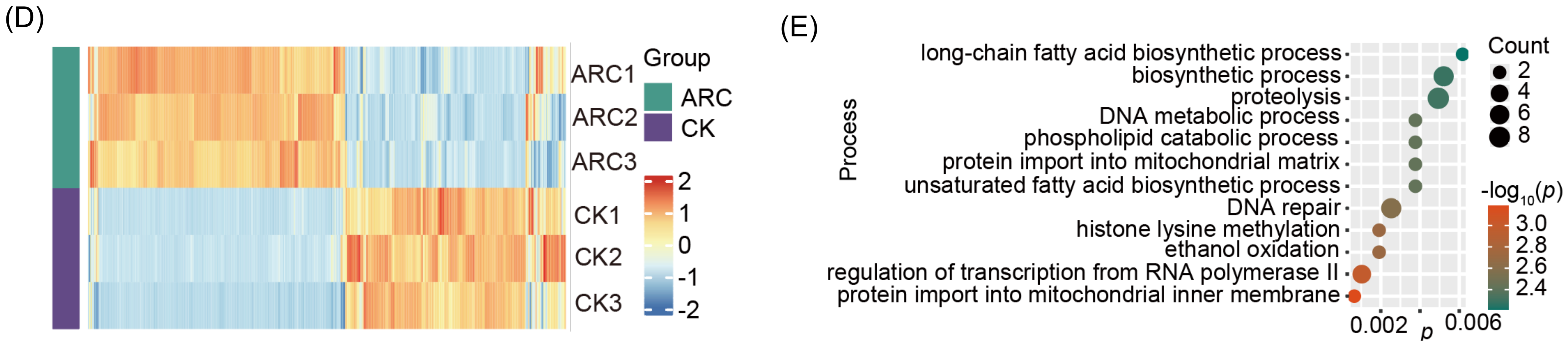


Figure 2 (D) Differentially expressed genes in ARC mediated inhibition of *A. flavus*. (E) GO enrichment analysis of genes involved in ARC-mediated inhibition of *A. flavus*.

ARC recruited rhizobia and activated their nodulation gene expression

- ARC fermentation supernatant tripled *Bradyrhizobium* colonies, indicating **rhizobia recruitment**
- Gene expression analysis showed that **ARC induced nodulation gene expression in rhizobia**

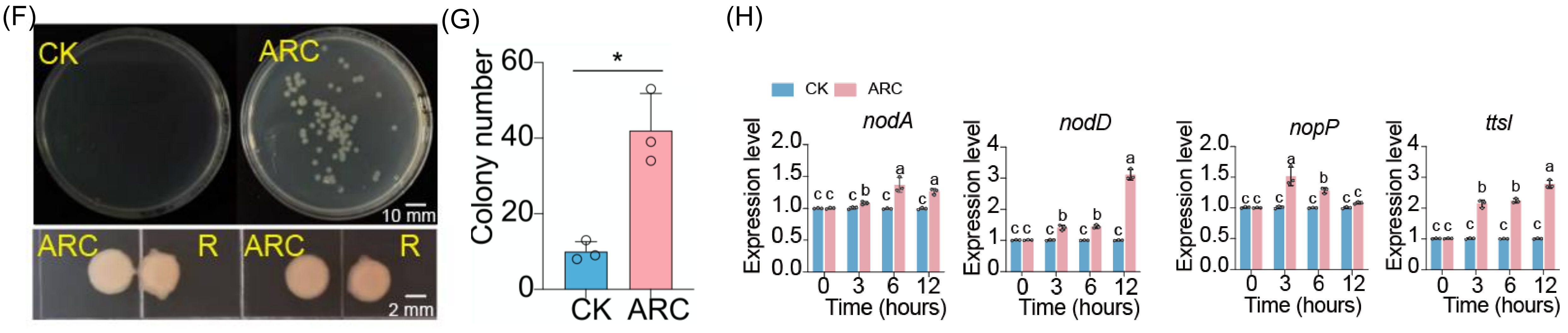


Figure 2 (F) Recruitment (top) and confrontation (bottom) assays showing ARC's effect on rhizobia. (G) Quantitative analysis of rhizobial recruitment. (H) Expression of rhizobial nodulation-related genes under ARC treatment.

ARC upregulated multiple nodulation genes in peanuts

- ARC significantly **upregulated 74 nodulation-related genes in peanuts**, including those involved in early infection, nodule nutrition, and metabolic functions – indicating that **sustained nodule formation** and **enhanced N_2 fixation capacity** are achieved

(I)

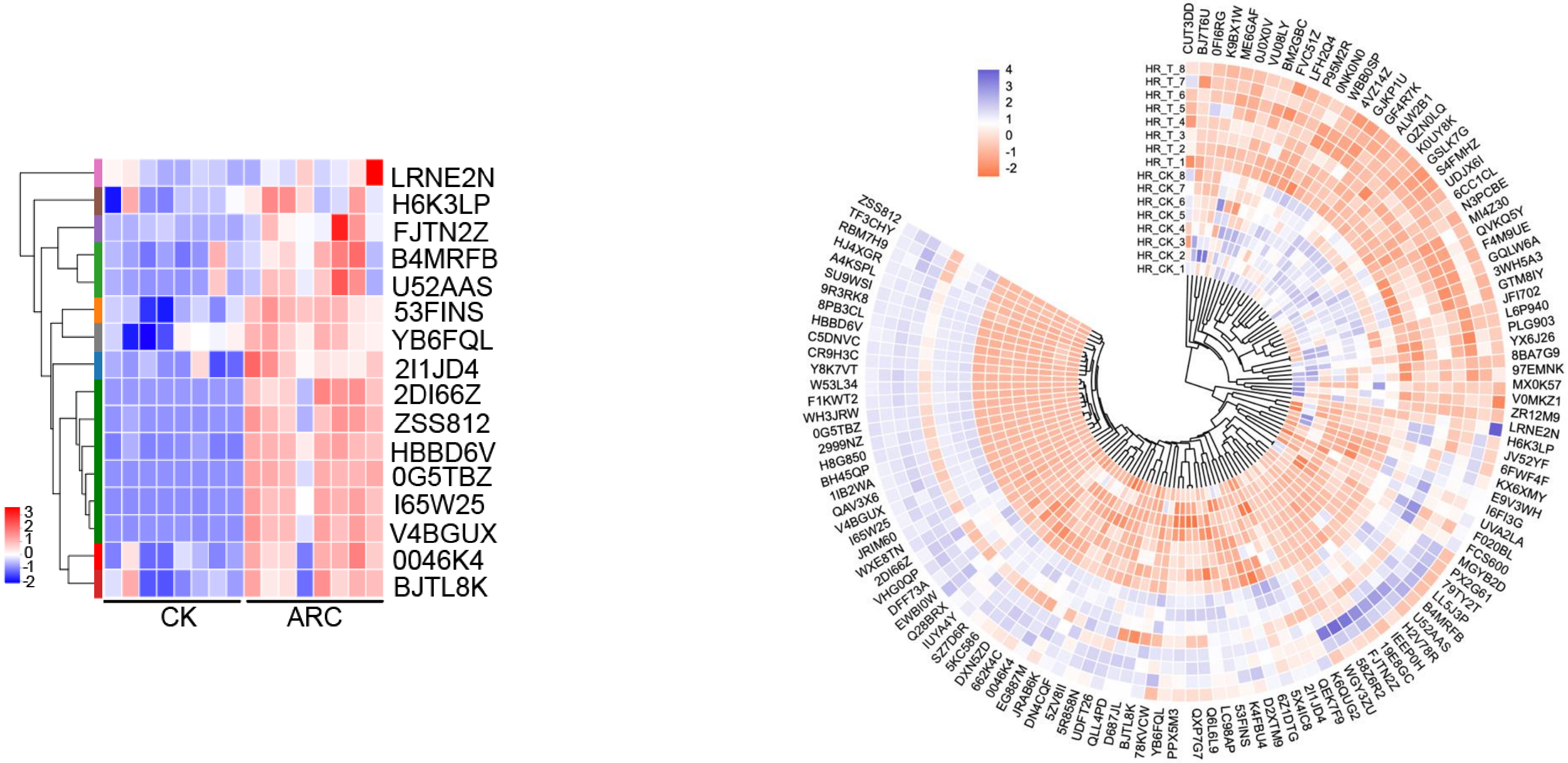


Figure 2 (I) Heatmap of nodulation-related gene expression in ARC-treated peanut roots

ARC enhanced peanut photosynthesis and carbon supply, forming a positive feedback loop

- ARC **reprogrammed carbon allocation dynamics** by upregulating SWEET family sugar transporter genes in roots and nodules, indicating enhanced carbon partitioning to the roots, formed a **powerful positive feedback loop**: increased carbon supply supports N_2 fixation, and N_2 fixation in turn promotes plant growth and photosynthesis

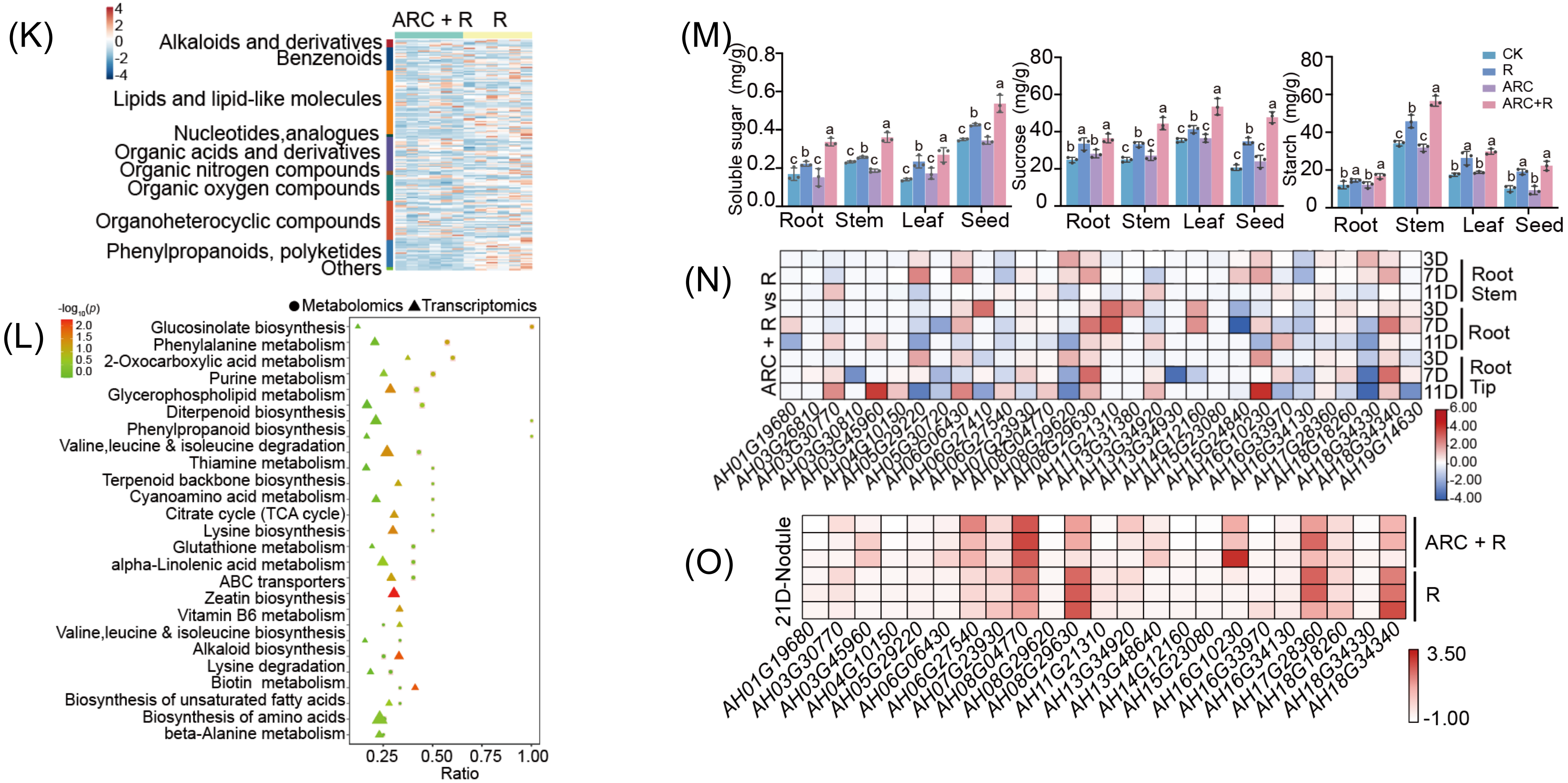


Figure2 (K) Heatmap of gene expression in peanut. (L) KEGG enrichment of differentially expressed genes and metabolites.(M) Sugar content across peanut tissues after ARC treatment. (N) Heatmap of SWEET gene expression in roots at early stages. (O) Heatmap of SWEET gene expression in nodules at later stages.

Summary

- In this study, we develop a novel strategy for exploring a dual-functional microbial synthetic community.
- Invent the SynCom ARC, which achieves aflatoxin control and rhizobia nodulation induction coupling in peanut.
- SynCom ARC inhibits *A. flavu*s growth and reduces peanut aflatoxin levels by 85.6% in 4 year field trials, enhances peanut nodulation and nitrogenase activity, retains active nodules at harvest, and boosts yield without super nodulation penalty in 325 sites of 19 provinces
- SynCom ARC inhibits multiple targets in *A. flavus*, recruits and activates nodulation and nitrogen fixation in rhizobia and peanut and improves photosynthesis and carbon supply for aflatoxin prevention and nodulation induction balancing yield increase.

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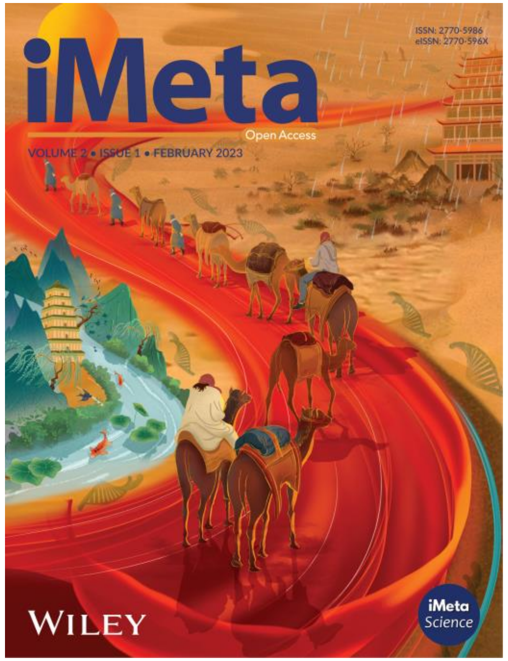

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