Trichoderma-Azotobacter biofilm as a promising inoculant for enhancing plant growth and soil nutrient dynamics in cotton

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Bioinoculant technology

- Microbial inoculants are an important component of integrated soil and crop management practices in agriculture
- For deriving maximum benefits from inoculation using microorganisms, effective colonisation of roots and rhizosphere is an essential step
- As several biotic and abiotic factors reduce the survival and proliferation of the applied bioinoculants in the rhizosphere
- **This necessitates alternative delivery method for bioinoculants**
- **Biofilmed biofertilizers are one of such recent innovation in agriculture**

Biofilms

- A biofilm is an assemblage or aggregation of microbial cells embedded in a self produced polymeric matrix
- Biofilm formation offers a reproductive fitness advantage in terms of slow growth and physiological heterogeneity, as compared to planktonic cells
- Mechanism to remain in a conducive place through effective colonization
- Strategy to overcome stress

Significance of biofilms in agriculture

- Several genera of agriculturally important (beneficial/pathogens) bacteria, fungi, cyanobacteria are reported to produce single or multispecies biofilms
- Biofilm formation on the plant roots is an important trait of rhizospheric microorganisms, which prevents them from being detached from the plant caused by various natural processes occurring in the soil
- Biofilm matrix makes the microbial cells more tolerant to stress conditions, thereby lengthens their survival rate in soils
- EPS also found to play an essential role in development of functional nodules in diazotrophs. Deletion of genes involved in biosynthesis of EPS resulted in formation of pseudonodules in several diazotrophs

Objectives

- To develop and evaluate the effect of fungus-bacterial biofilm on root and rhizosphere colonisation

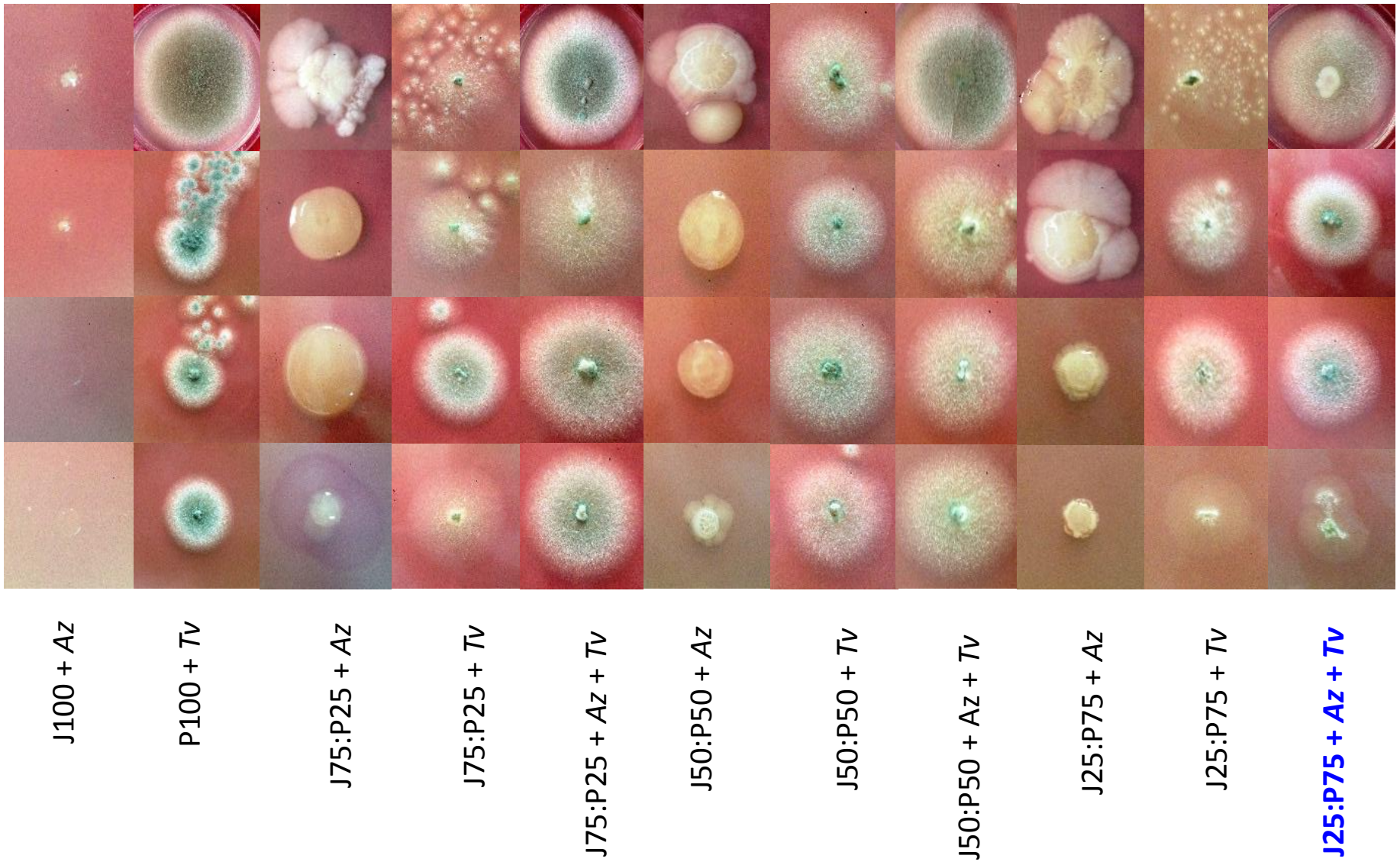
- To understand the role of fungus-bacterial biofilm on plant growth, soil nutrient availability, soil biology and plant defense enzymes in cotton

Microbial partners used in the study

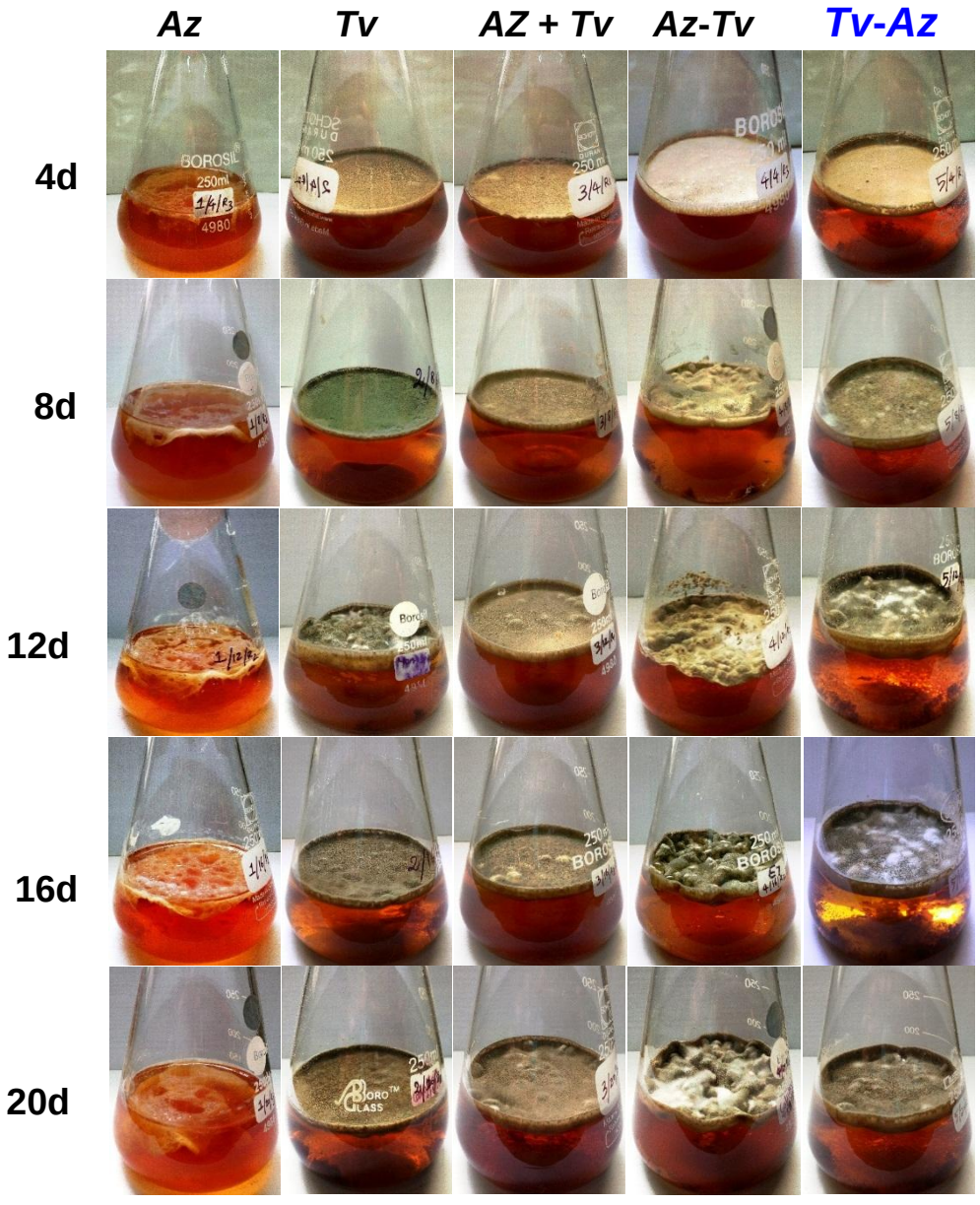
Azotobacter chroococcum
(MTCC 25045/NAIMCC-B-00061)

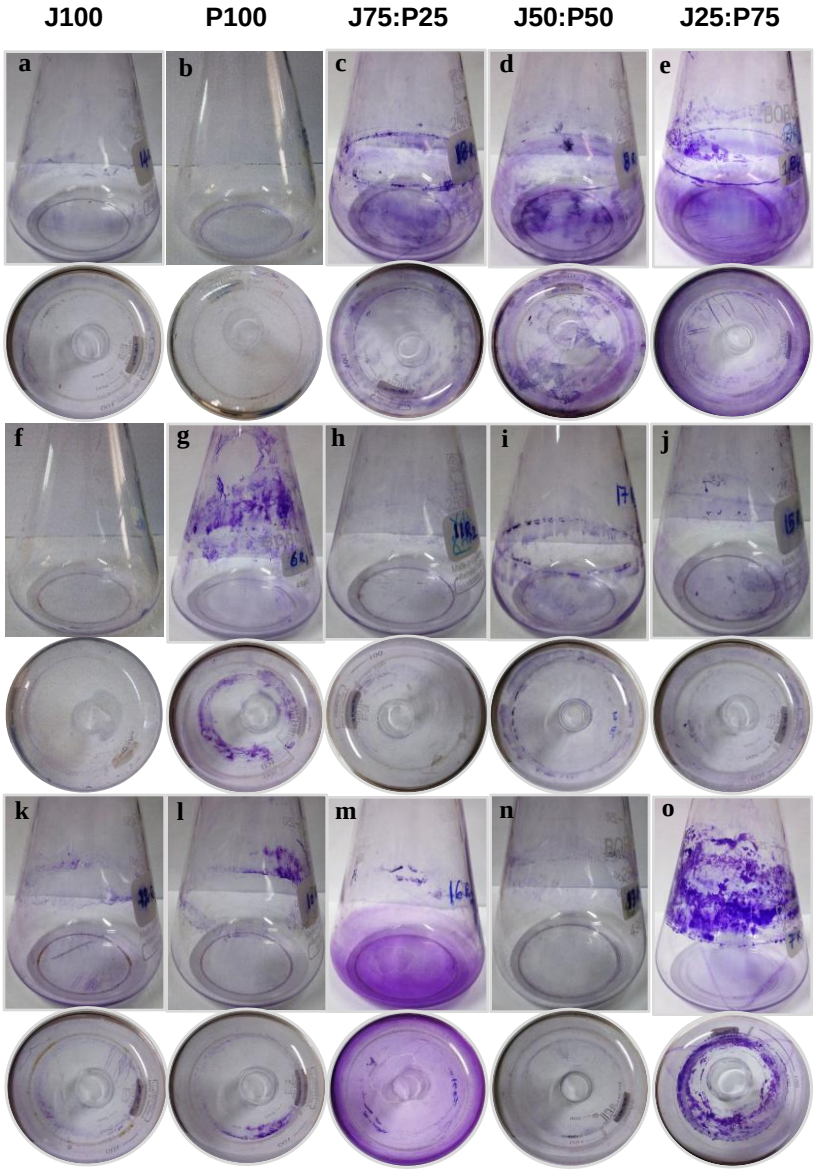
Trichoderma viride
(ITCC 2211)

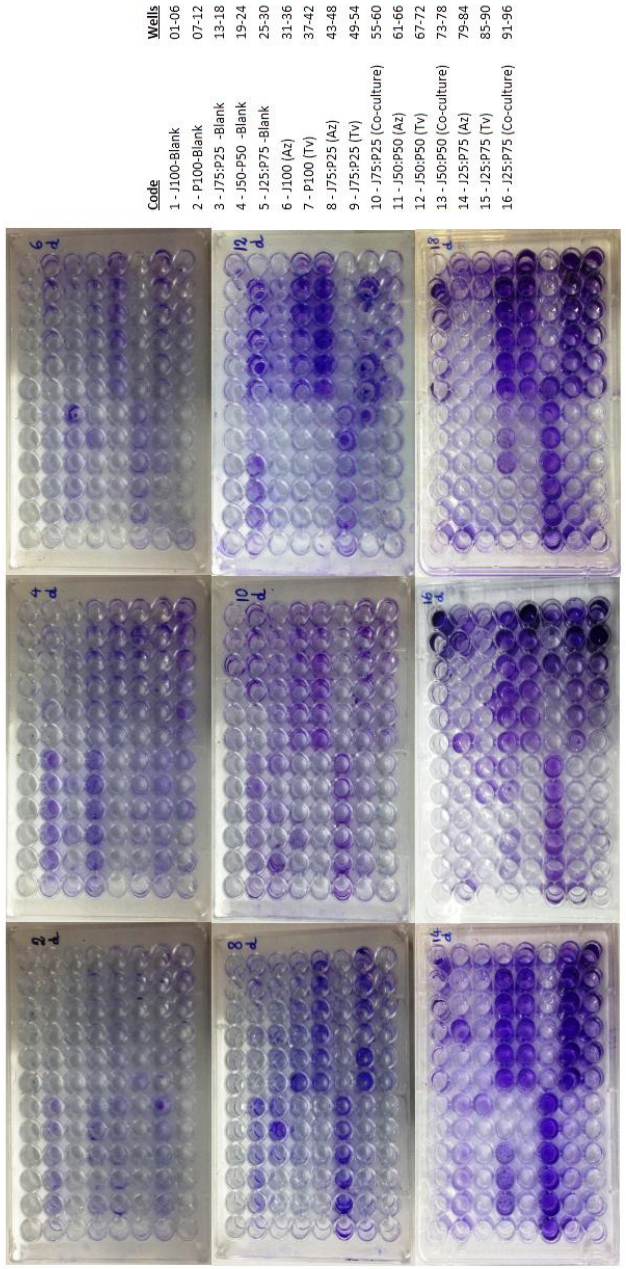
Colony characteristics of *A. chroococcum*, *T. viride* and their biofilm in different growth media ratios (4-16 days)



Biofilm development under individual, coculture and staggered inoculation

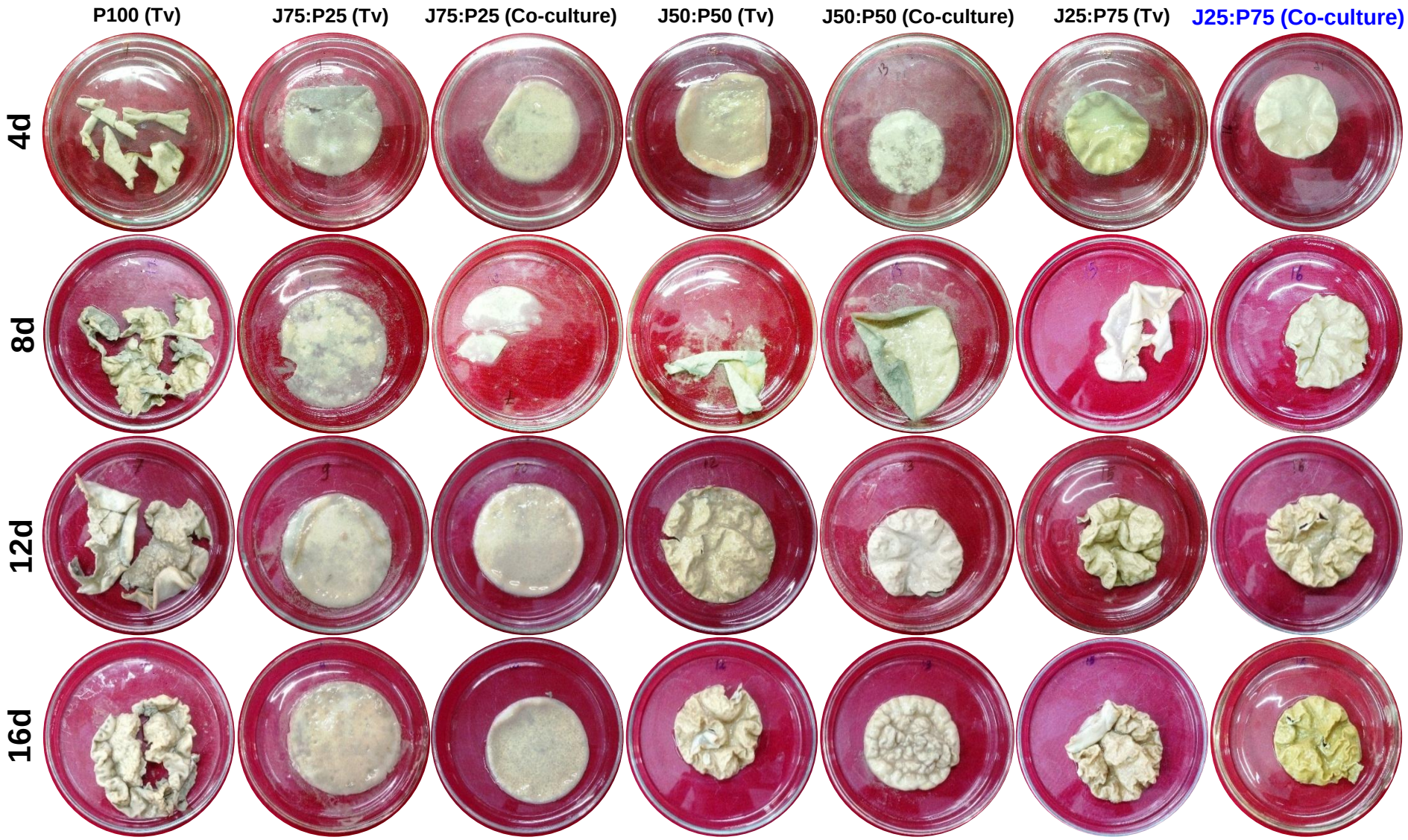




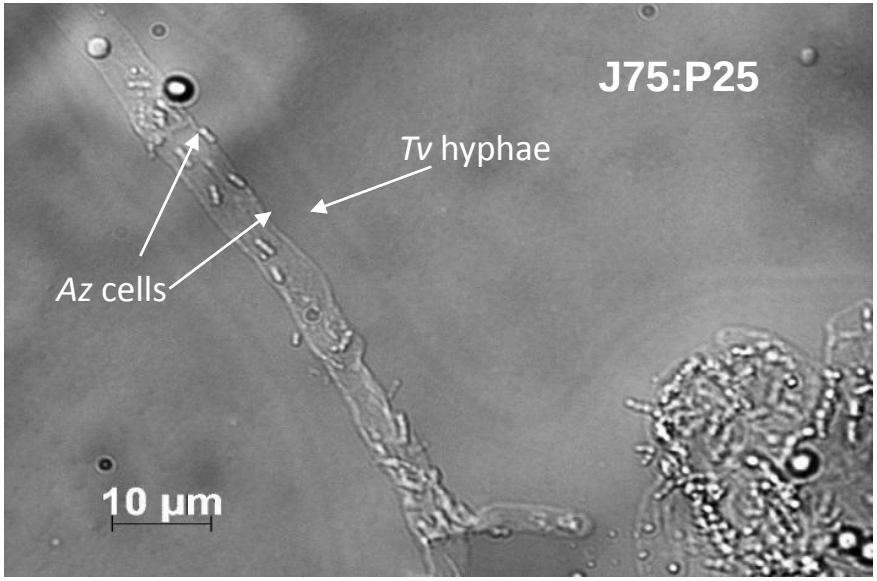


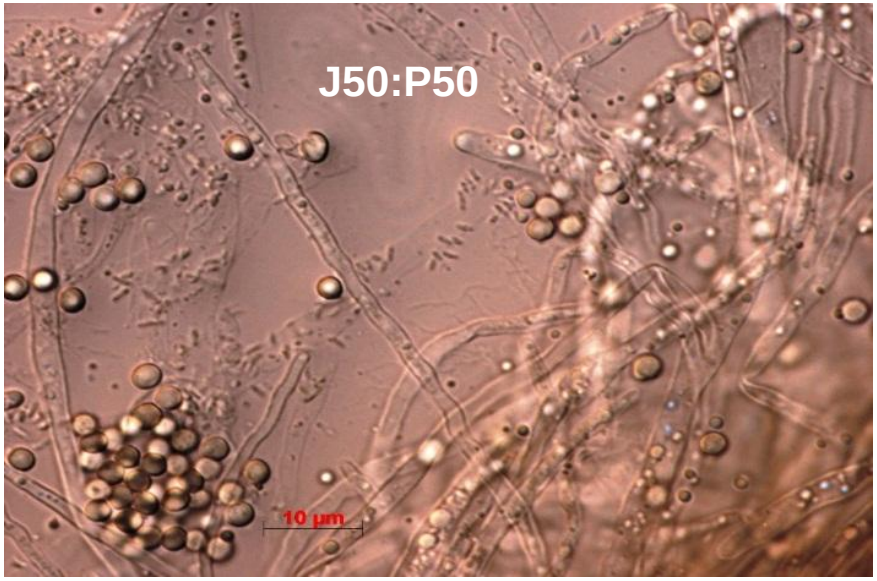
Quantification of biofilm formation in different growth media, using crystal violet assay after 16 days of inoculation. **a**, J100 - *A. chroococcum*; **b**, P100 - *A. chroococcum*; **c**, J75:P25 - *A. chroococcum*; **d**, J50:P50 - *A. chroococcum*; **e**, J25:P75 - *A. chroococcum*; **f**, J100 - *T. viride*; **g**, P100 - *T. viride*; **h**, J75:P25 - *T. viride*; **i**, J50:P50 - *T. viride*; **j**, J25:P75 - *T. viride*; **k**, J100 - *A. chroococcum- T. viride* co-culture; **l**, P100 - *A. chroococcum- T. viride* co-culture; **m**, J75:P25 - *A. chroococcum- T. viride* co-culture; **n**, J50:P50 - *A. chroococcum-T. viride* coculture; **o, J25:P75 - *A. chroococcum- T. viride* co-culture**

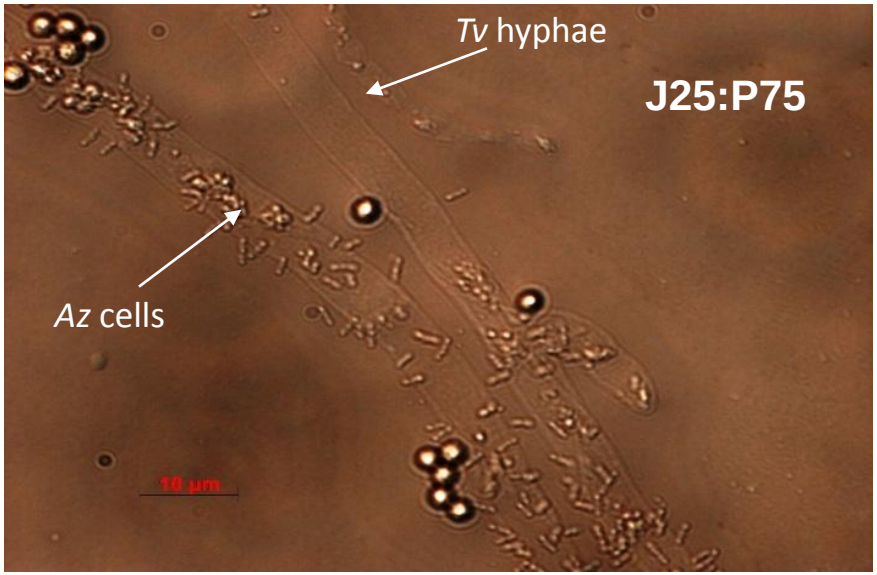
Biofilm biomass in different growth media



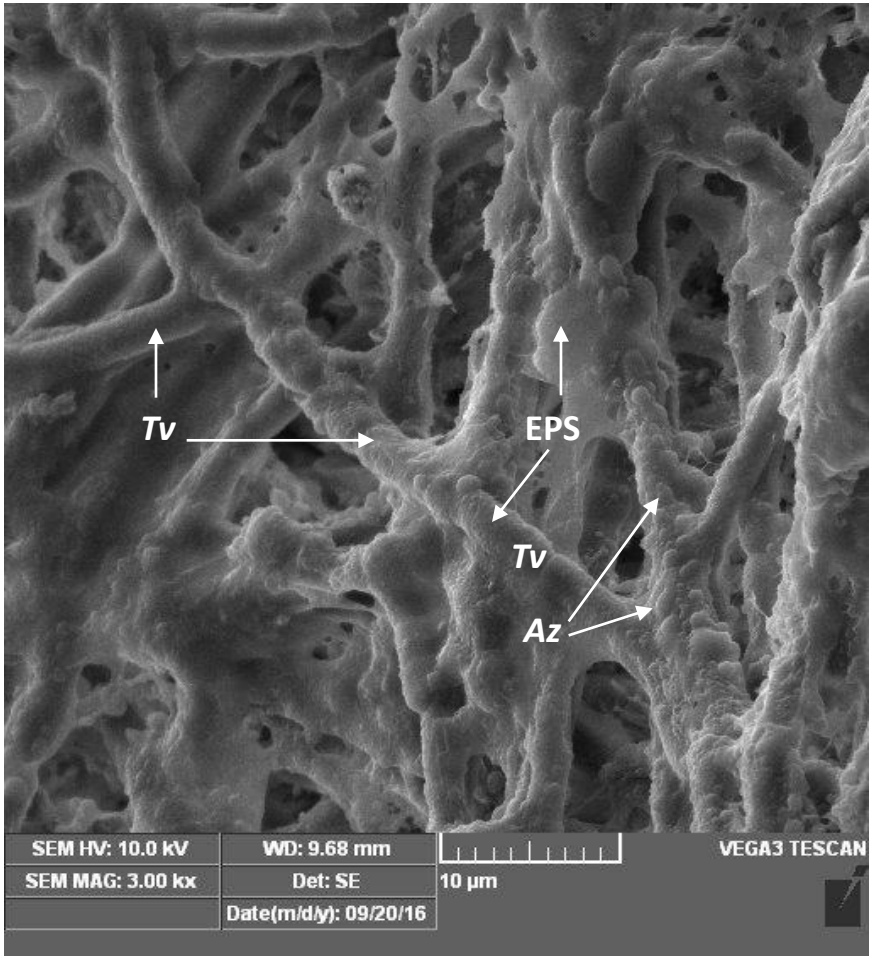
Phase contrast microphotographs of coaggregation between *A. chroococcum-T. viride* – 16 dpi

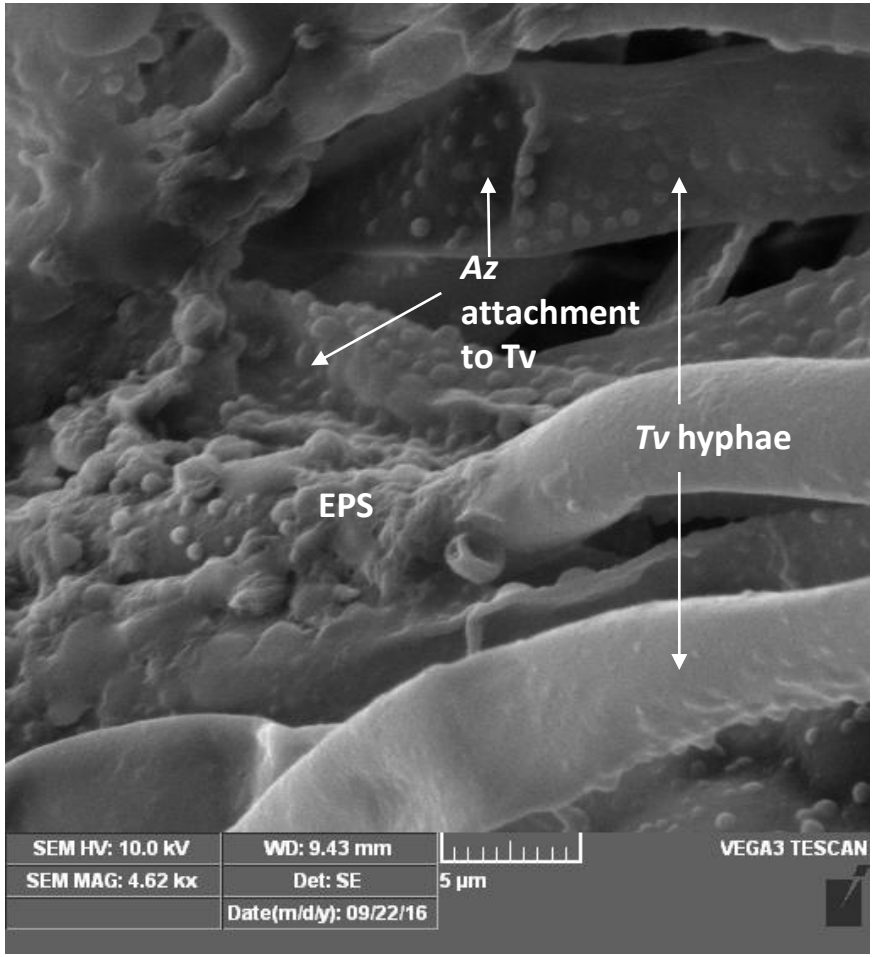






Scanning electron microphotograph of *Tv-Az* biofilm





Root colonization study

National Phytotron Facility

- To understand the colonization behavior of developed biofilms in the rhizosphere/rhizoplane of cotton

Cotton (Suraj)

Surface-sterilized in a 1% sodium hypochlorite solution for 5 min, washed five times with sterile water for 3 min each, and soaked in sterile water overnight

Treatment details

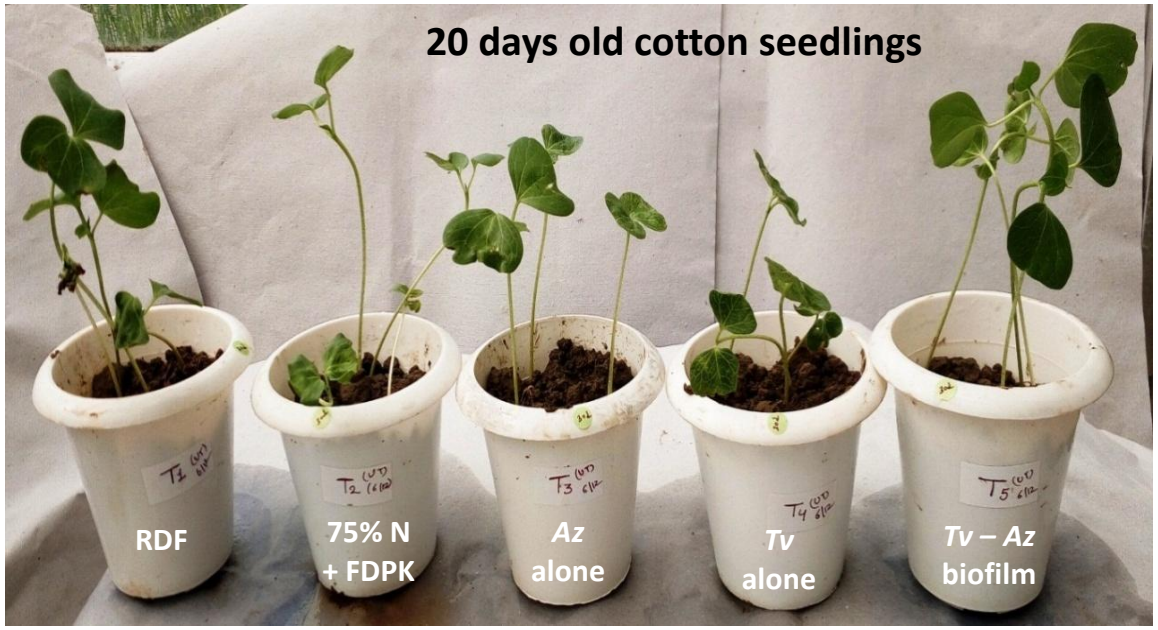
T1: Recommended dose of fertilizers (RDF) - no microbial inoculation
100 kg N (urea): 50 kg P_2O_5 (SSP): 50 kg K_2O (MOP) ha^{-1}
T2: 75% Nitrogen (N) + Full dose of Phosphorus (P) and Potassium (K) (75% N + FDPK) - no microbial inoculation
T3: 75% N + FDPK + *A. chroococcum* (*Az*)

T4: 75% N + FDPK + *T. viride* (*Tv*)

T5: 75% N + FDPK + *T. viride – A. chroococcum* biofilm (*Tv-Az*)

The cotton seeds were inoculated with 0.5 mL of *Az* (10^{10} cfu mL^{-1}, 48h old culture), *Tv* (10^8 spores mL^{-1}, 1 week old culture), and *Tv-Az* biofilm (Az:10^{10} cfu mL^{-1} and *Tv* :10^{10} spores mL^{-1}, 6d old biofilm)

Biofilm on growth characteristics of 20d old cotton seedlings



Evaluation of microbial inoculation on plant growth parameters (20 DAI)

Treatments	Seedlings length (cm) *		Seedlings fresh wt. (mg) *		Seedlings dry wt. (mg) *		Root: Shoot ratio*	Protein (mg g^{-1} fresh wt.)**	
	Shoot	Root	Shoot	Root	Shoot	Root		Leaves	Roots
RDF	14.0^{c}	9.2^{a}	1740^{b}	0.260^{b}	0.650^{b}	0.210^{d}	0.33^{b}	6.2^{c}	3.7^{c}
75%N+FDPK	13.6^{c}	7.9^{b}	1170^{c}	0.180^{c}	0.570^{c}	0.190^{e}	0.33^{b}	5.9^{d}	3.5^{c}
75%N+FDPK + *Az*	15.8^{b}	9.9^{a}	1860^{ab}	0.280^{b}	0.680^{b}	0.250^{b}	0.37^{a}	7.4^{b}	5.7^{b}
75%N+FDPK + *Tv*	14.3^{c}	9.6^{a}	1770^{b}	0.270^{b}	0.650^{b}	0.250^{c}	0.37^{a}	7.2^{b}	5.9^{b}
75%N+FDPK + *Tv-Az*	**17.2^{a}**	**10.1^{a}**	**2120^{a}**	**0.320^{a}**	**0.750^{a}**	**0.300^{a}**	**0.39^{a}**	**10.2^{a}**	**7.0^{a}**
cv	6.52	5.37	11.5	11.2	4.72	1.95	5.00	1.89	2.93
SEm ±	0.32	0.20	0.07	0.01	0.01	0.007	0.006	0.51	0.45
CD (p=0.05)	1.28	0.87	0.26	0.03	0.04	0.006	0.02	0.36	0.38

* n=10; ** n=3

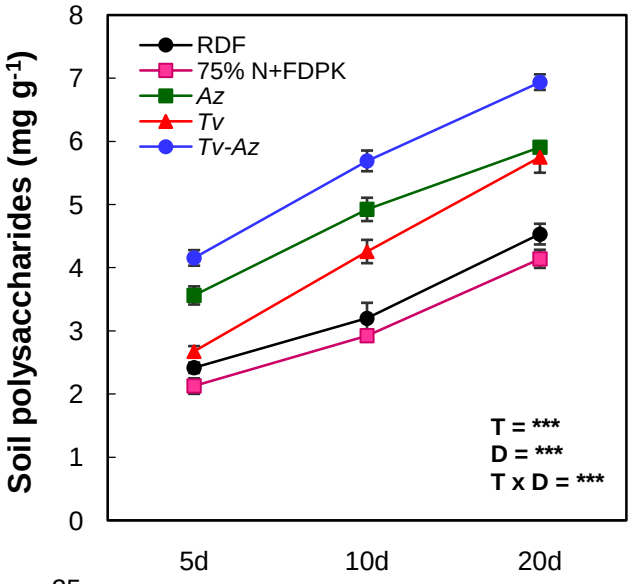
The *Tv-Az* biofilm inoculation showed an increase of **23% and 10%** in terms of shoot and root length respectively, over the RDF

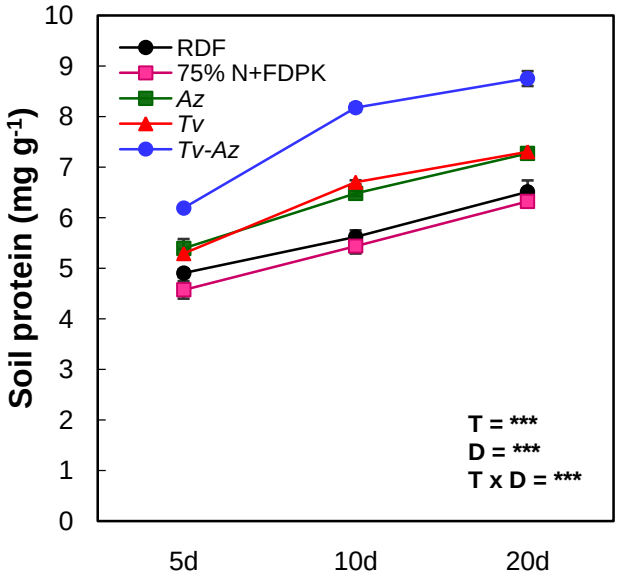
Effects of microbial inoculation on macro- and micronutrient status of cotton grown soil (20 DAI)

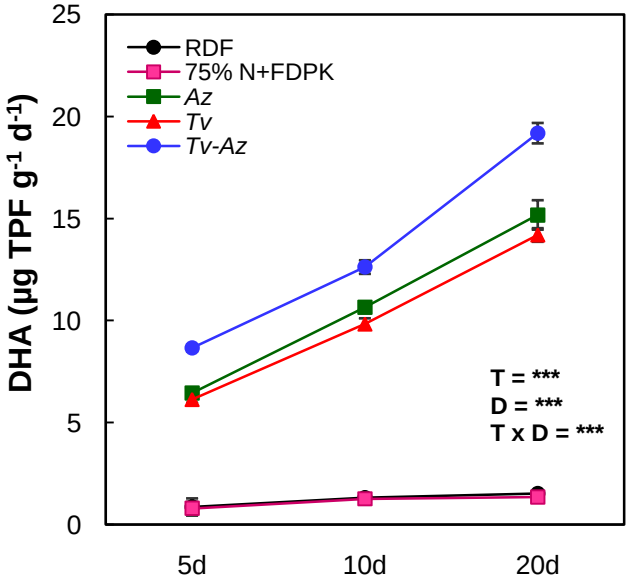
Treatments	OC (%)	Available N (mg kg^{-1})	Available P (mg kg^{-1})	Available K (mg kg^{-1})	Zinc (mg kg^{-1})	Copper (mg kg^{-1})	Manganese (mg kg^{-1})	Iron (mg kg^{-1})
RDF	0.52 ± 0.02^{bc}	56 ± 0.68^{c}	3.0 ± 0.52^{cd}	83 ± 1.42^{bc}	2.5 ± 0.56^{b}	1.6 ± 0.10^{cd}	1.1 ± 0.20^{c}	1.5 ± 0.09^{c}
75%N+FDPK	0.49 ± 0.01^{c}	51 ± 0.84^{e}	2.4 ± 0.52^{d}	79 ± 0.89^{d}	1.2 ± 0.16^{d}	1.4 ± 0.05^{d}	0.8 ± 0.10^{c}	1.3 ± 0.04^{d}
75%N+FDPK + *Az*	0.56 ± 0.03^{ab}	60 ± 1.12^{b}	3.9 ± 0.52^{bc}	86 ± 2.34^{b}	2.7 ± 0.20^{b}	1.9 ± 0.26^{c}	1.5 ± 0.04^{b}	1.5 ± 0.05^{c}
75%N+FDPK + *Tv*	0.53 ± 0.01^{bc}	54 ± 1.57^{d}	4.8 ± 0.52^{b}	81 ± 0.34^{cd}	1.9 ± 0.14^{c}	2.3 ± 0.26^{b}	1.7 ± 0.29^{b}	1.7 ± 0.06^{b}
75%N+FDPK + *Tv-Az*	**0.60 ± 0.04^{a}**	**63 ± 0.34^{a}**	**6.0 ± 1.03^{a}**	**90 ± 2.27^{a}**	**3.5 ± 0.28^{a}**	**2.7 ± 0.16^{a}**	**2.2 ± 0.13^{a}**	**1.9 ± 0.03^{a}**
cv	4.71	1.76	16.3	1.96	12.9	9.44	12.0	3.58
SEm ±	0.01	1.13	0.36	1.11	0.22	0.13	0.13	0.05
CD (p=0.05)	0.04	1.82	1.18	2.99	0.55	0.34	0.32	0.10

- 50% increase in SOC in *Tv-Az* biofilm treatment as compared to their initial values
- 30% increase in soil available nitrogen, 3.6 fold increase in P and 18% increase in K in *Tv-Az* biofilm treatment as compared to their initial values
- *Tv-Az* treatment showed a fold increase of 3.2 (Zn), 1.9 (Cu), 3.5 (Mn) and 2.5 (Fe) over the initial micronutrient status

Time course changes in soil polysaccharides, proteins and dehydrogenase activity as influenced by microbial inoculation



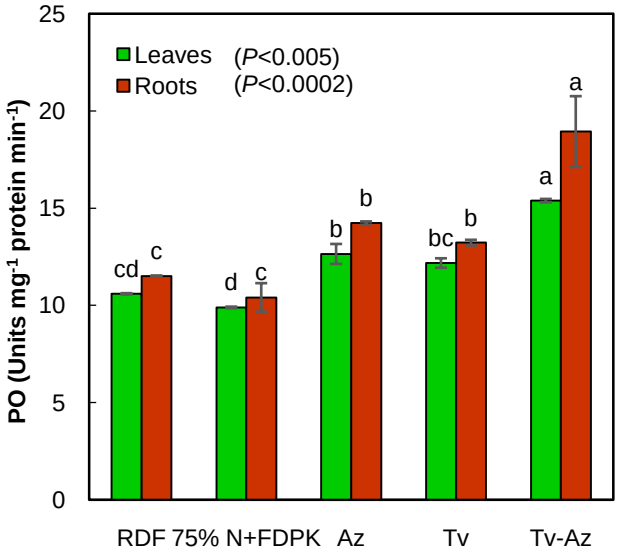


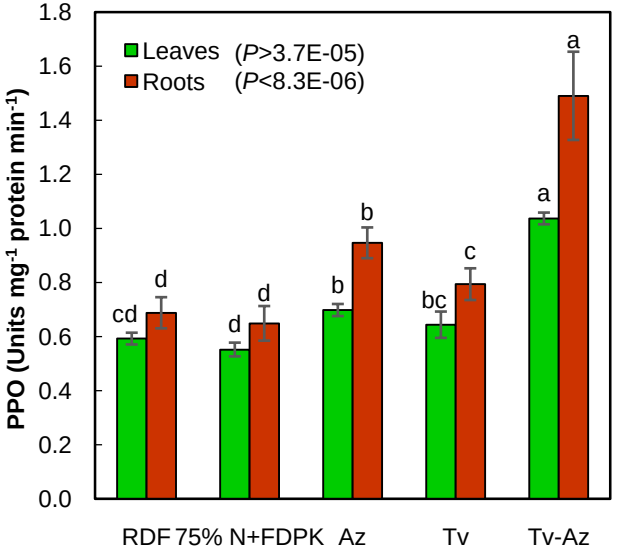


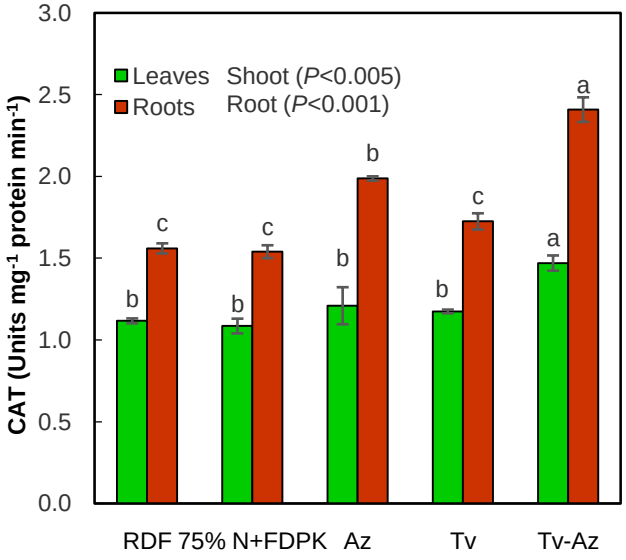
- 4.4 fold increase in soil polysaccharide content
- 4.3 fold increase in soil protein content
- Several fold increase in DHA

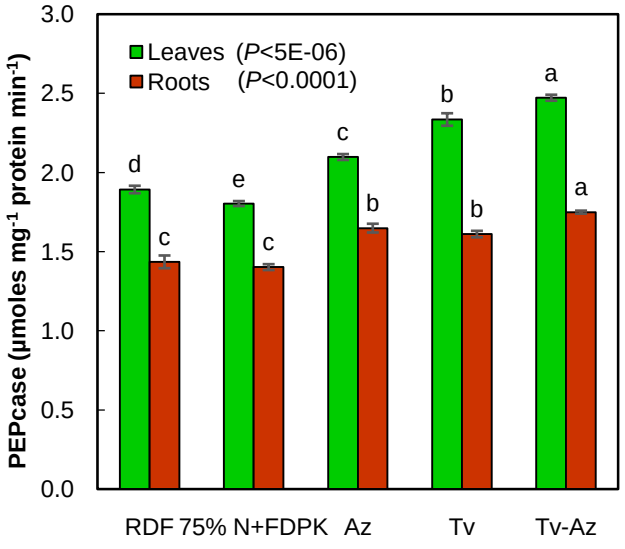
in *Tv-Az* treatment as compared to the initial soil values

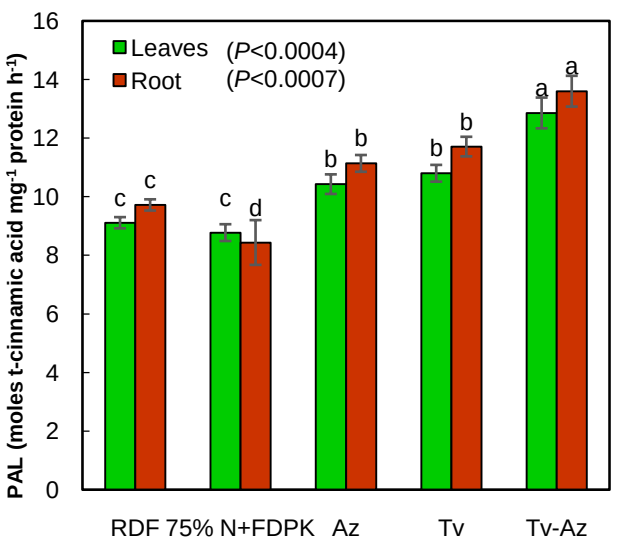
Effect of microbial inoculation on plant antioxidant and defense enzymes activities in 20d old cotton seedlings

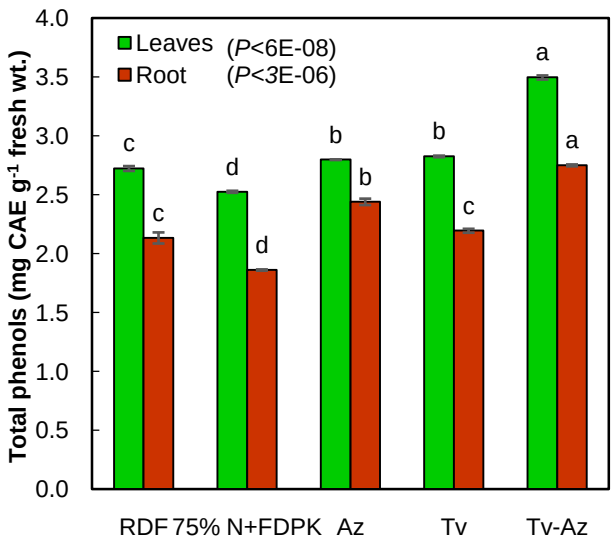




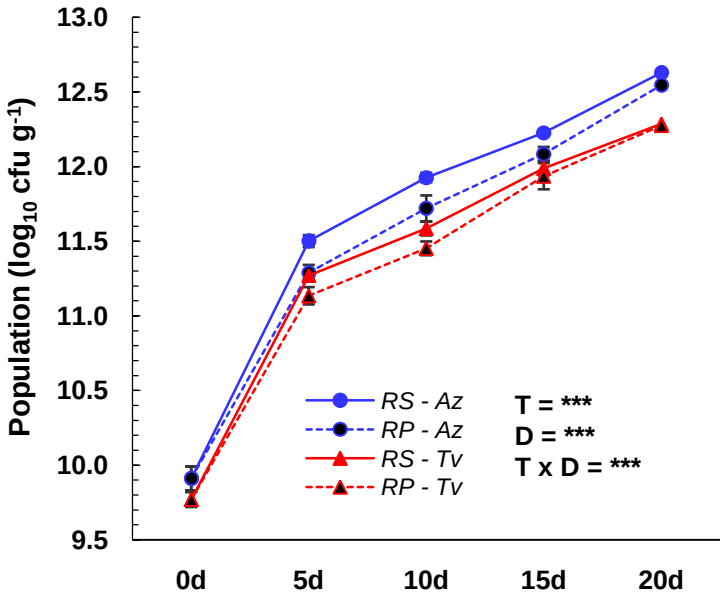


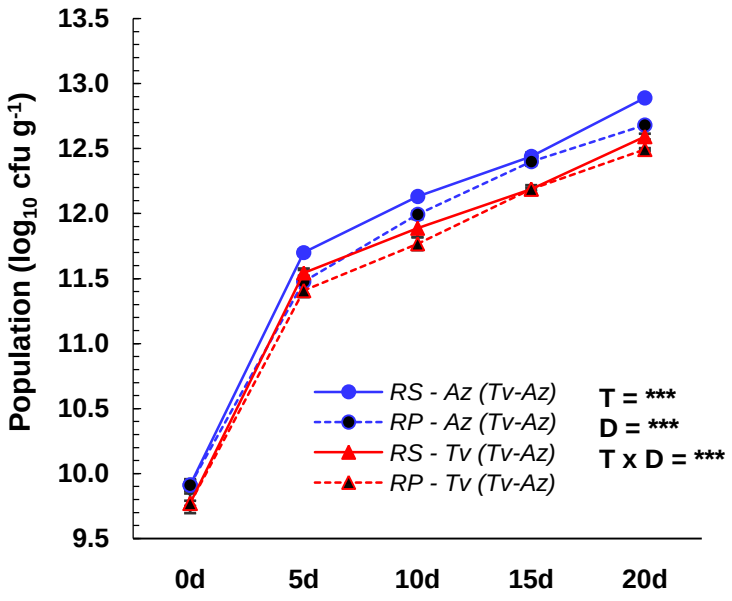






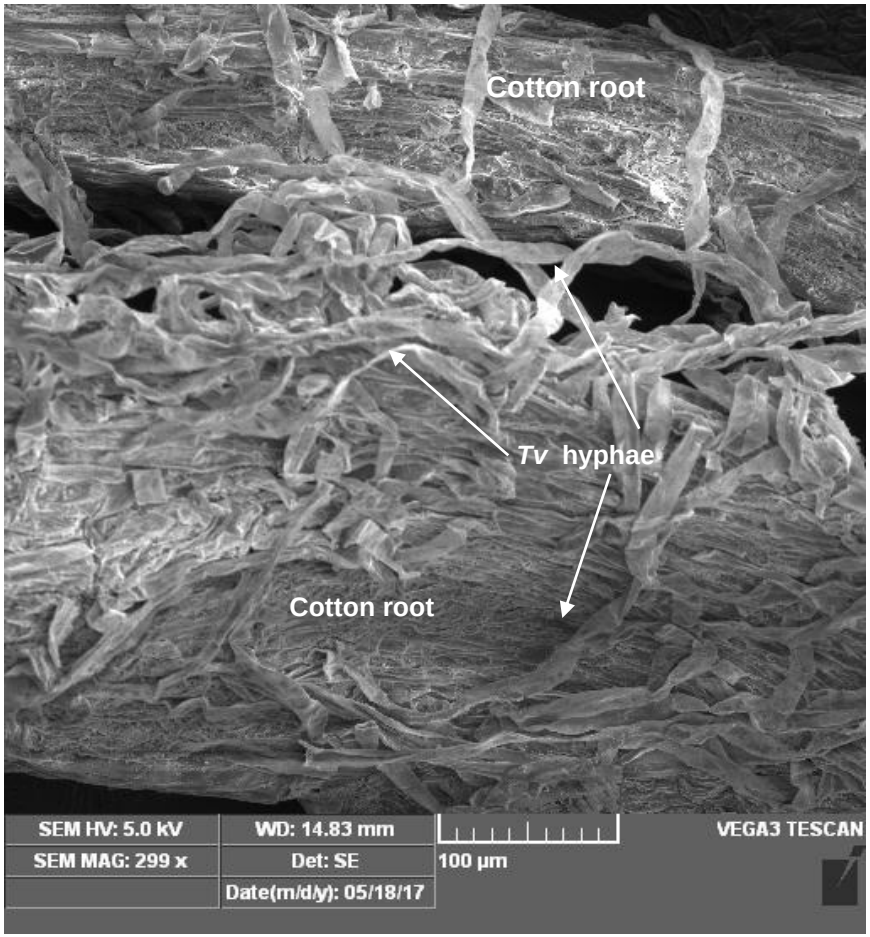
Time course analyses of colonization behaviour of microbial inoculants in cotton rhizosphere and rhizoplane

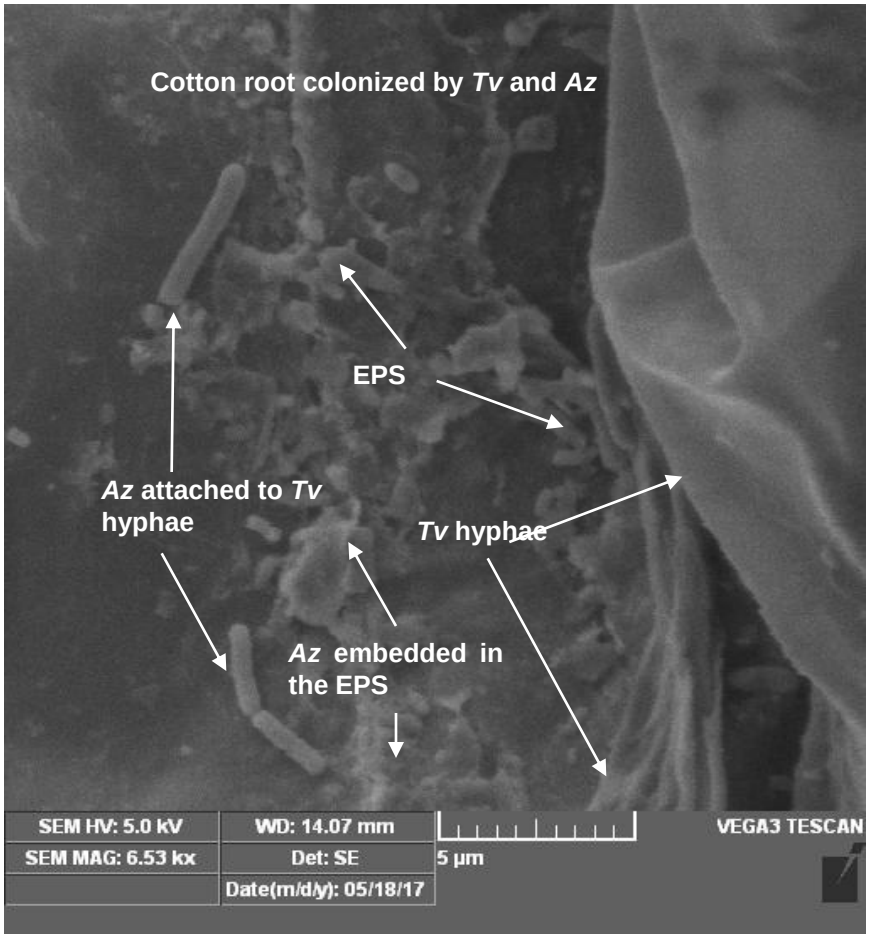




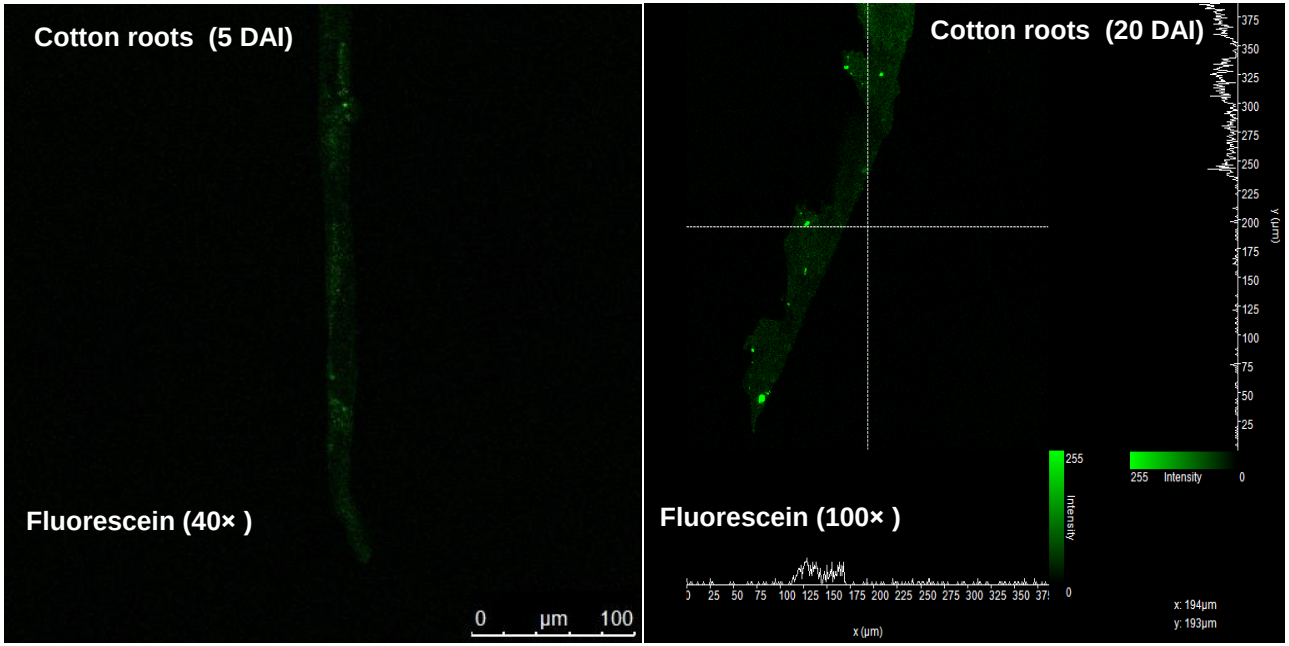
- Rhizosphere population found to be significantly (p<0.0001) higher as compared to the rhizoplane population density
- The population densities of *Tv-Az* biofilm in the rhizosphere and rhizosphere were found to be significantly higher in the biofilm treatment
- As compared to *Az* population (rhizosphere: 2% and rhizoplane: 1%) in *Tv-Az* biofilm, the *Tv* treatment showed increased population densities in both rhizosphere (2.49%) and rhizoplane (1.79%), as compared to their single inoculation

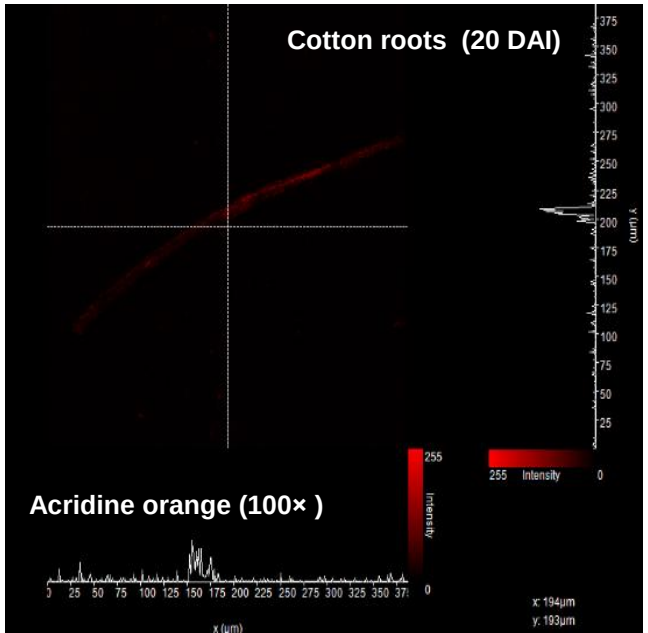
Scanning electron microphotographs depicting cotton root colonisation by *Tv-Az* biofilm



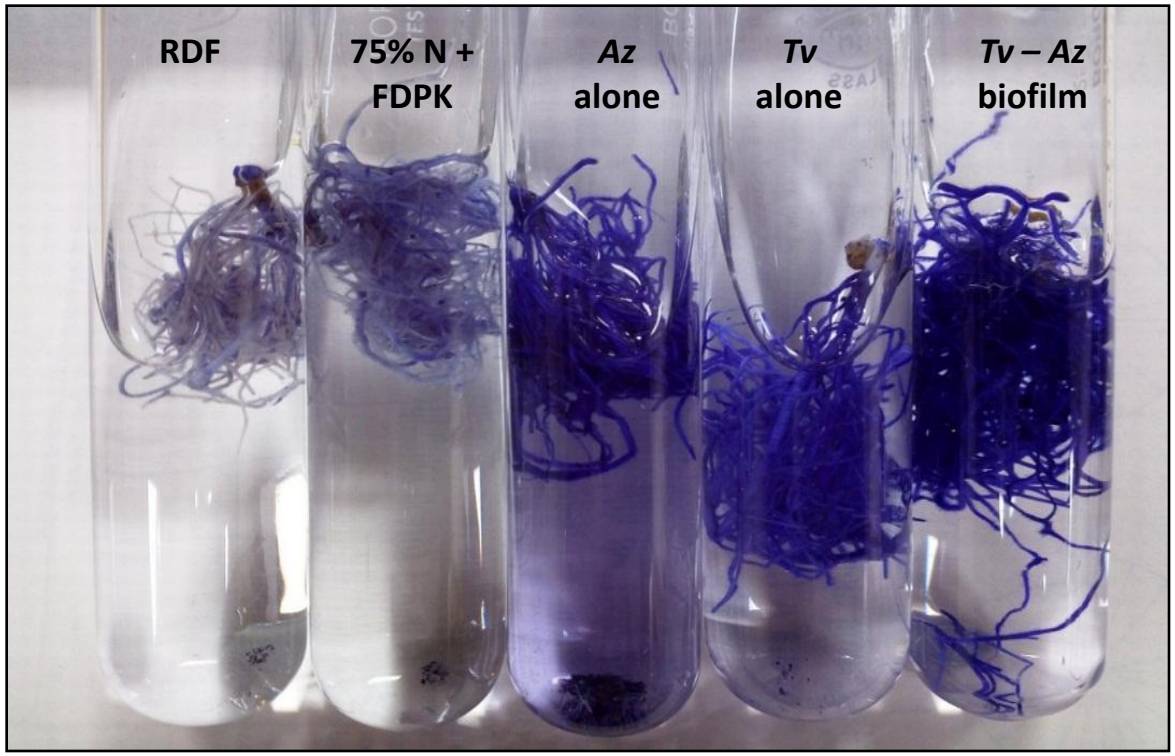


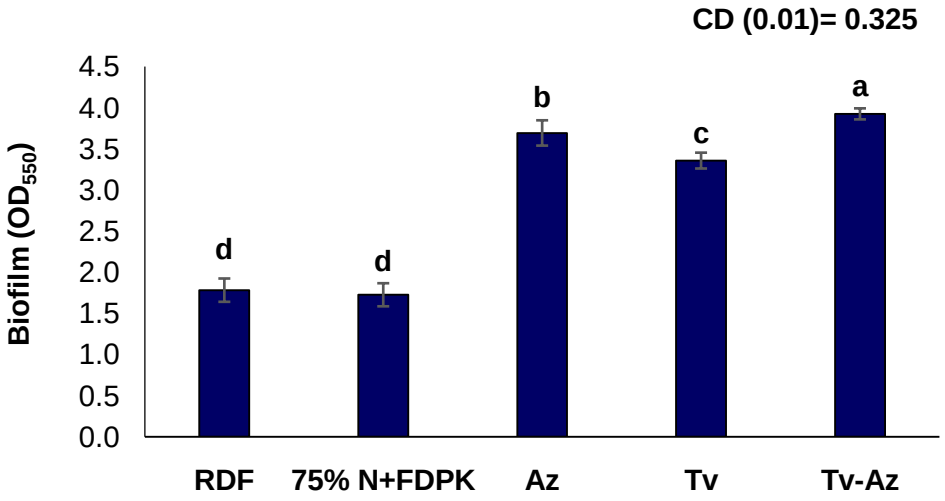
Confocal laser scanning microscopy depicting cotton root colonisation by *Tv-Az* biofilm





Quantification of biofilm formation through crystal violet staining





Transcriptome analysis

- Transcriptome analysis was undertaken to understand the transcriptional (downregulated and upregulated genes) responses in *A. chroococcum* cells *and T. viride* vs *Tv-Az* biofilm

RNASeq high quality data statistics of *A. chroococcum, T. viride* and *Tv-Az* biofilm

	Azotobacter chroococcum (Az)		*Trichoderma viride* (Tv)		*Tv-Az* biofilm	
	R1	R2	R1	R2	R1	R2
No. of reads	**7,898,059**	**7,362,242**	**15,547,359**	**16,231,968**	**33,683,972**	**30,356,072**
No. of bases	1,188,251,803	1,107,059,972	2,326,627,601	2,437,036,277	5,062,473,622	4,559,608,730
Total data (Gb)	1.18	1.10	2.32	2.43	5.06	4.55

Assembly statistics of *A. chroococcum* and *T. viride*

	Azotobacter chroococcum		*Trichoderma viride*	
	R1	R2	R1	R2
No. of transcripts	**6432**	**30419**	**983**	**43330**
Total bases	1,712,320	19,236,033	345,094	29,407,013
Mean transcript length	266	632	351	678
N50	248	978	333	1237
Max. transcript length	3159	8631	3314	10057
Min. transcript length	201	201	201	201

Coding sequence (CDS) statistics of *A. chroococcum* and *T. viride*

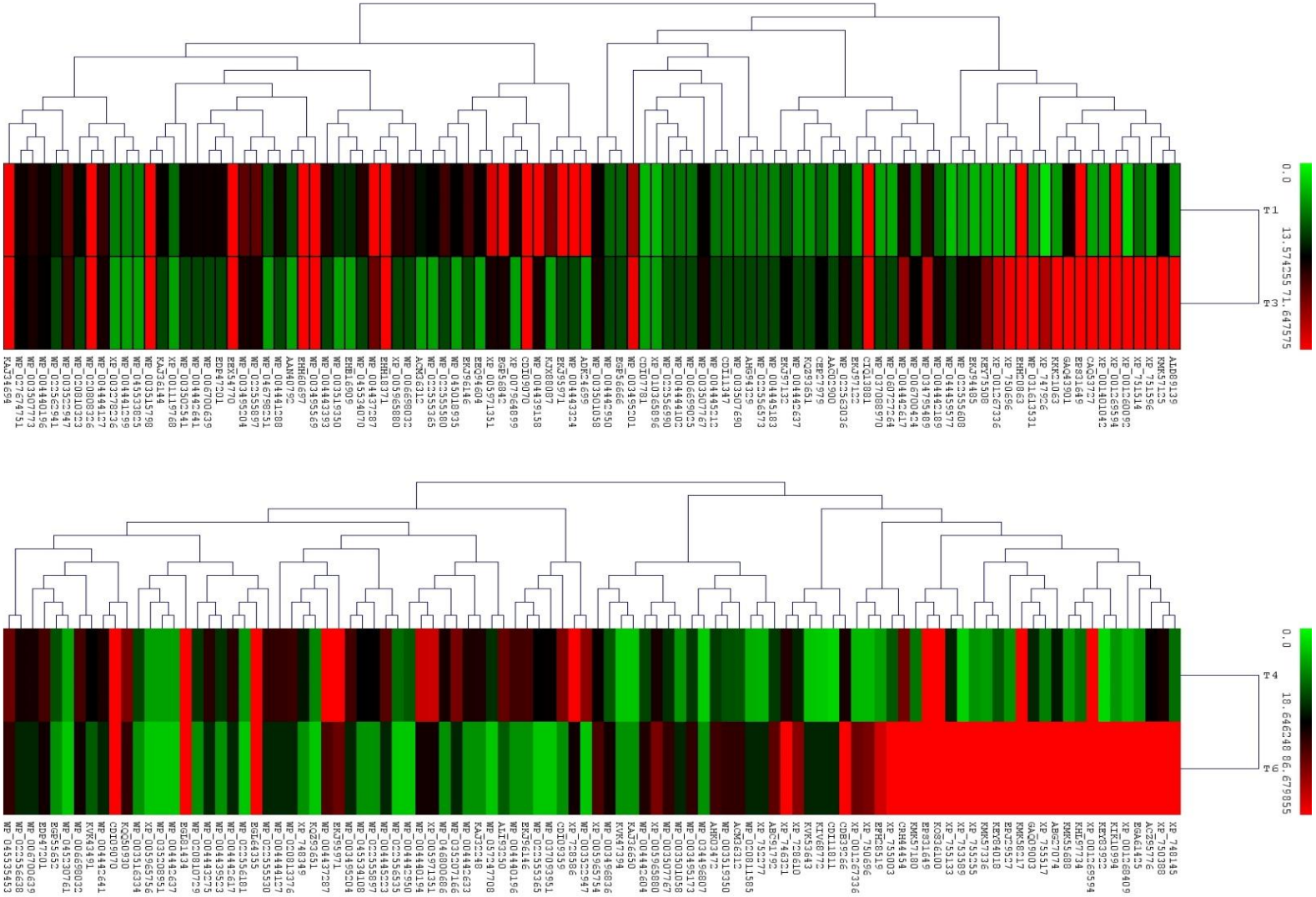
	Azotobacter chroococcum		*Trichoderma viride*	
	R1	R2	R1	R2
No. of CDS	**538**	**322**	**13,314**	**16,606**
Total bases	23,037	152,703	10,020,066	15,176,673
Mean CDS length	428	474	752	913
Max. CDS length	1,980	2,226	7,956	9,381
Min. CDS length	297	297	297	297

Gene ontology (GO) distribution for CDS of *A. chroococcum* and *T. viride*

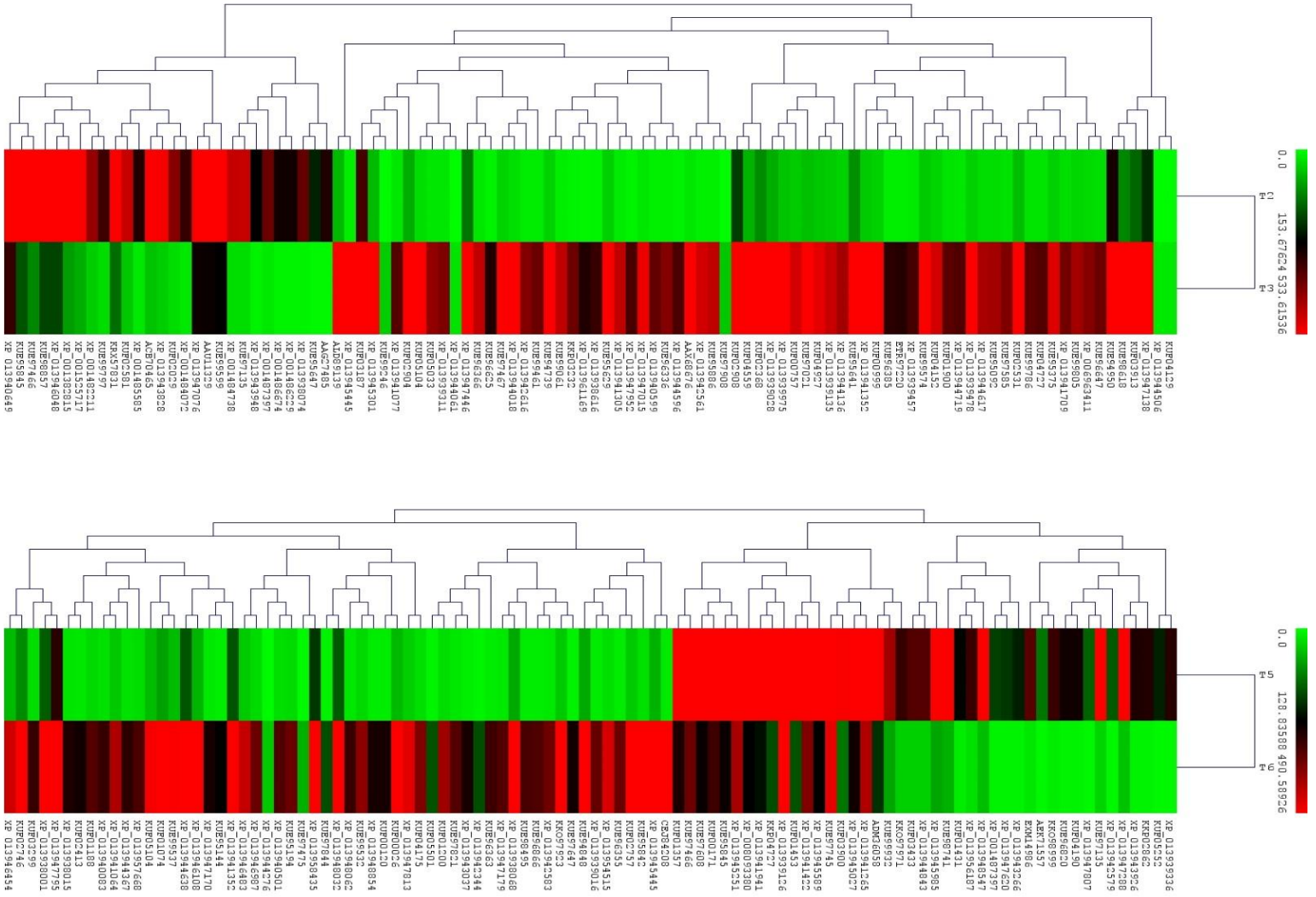
Sample	Biological processes	Molecular functions	Cellular component
A. chroococcum -R1	244	247	186
A. chroococcum -R2	170	178	117
T. viride -R1	6519	7002	5205
T. viride -R2	7721	8390	6121

Statistics of differentially expressed CDS of *A. chroococcum* and *T. viride*

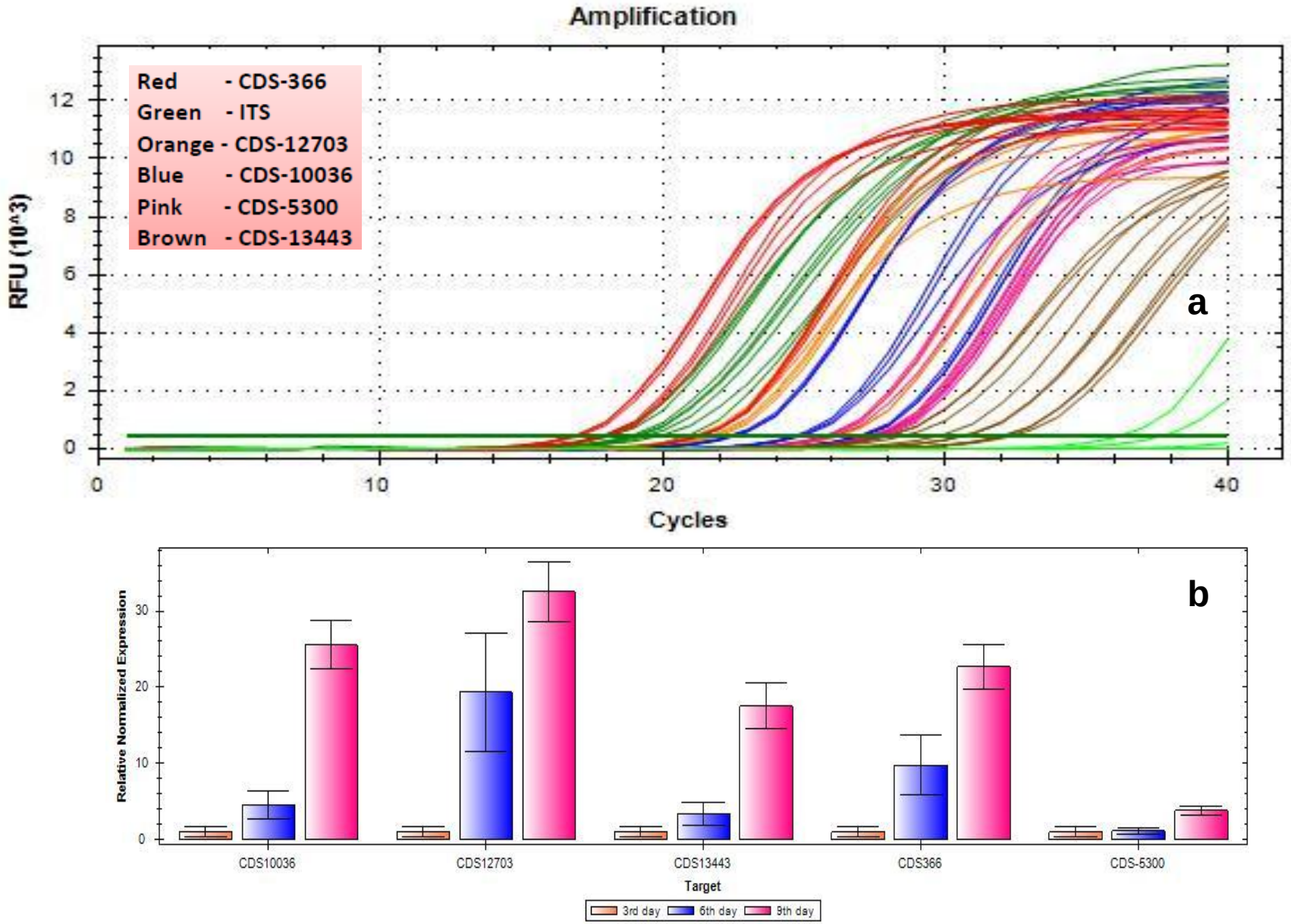
Sample	**Commonly expressed**	**Up-regulated**	**Down-regulated**
A. chroococcum R1 Vs. Tv-Az biofilm R1	115	59	56
A. chroococcum R2 Vs. Tv-Az biofilm R2	153	76	77
T. viride R1 Vs. Tv-Az biofilm R1	**8222**	**4114**	**4108**
T. viride R2 Vs. Tv-Az biofilm R2	**9166**	**4629**	**4537**



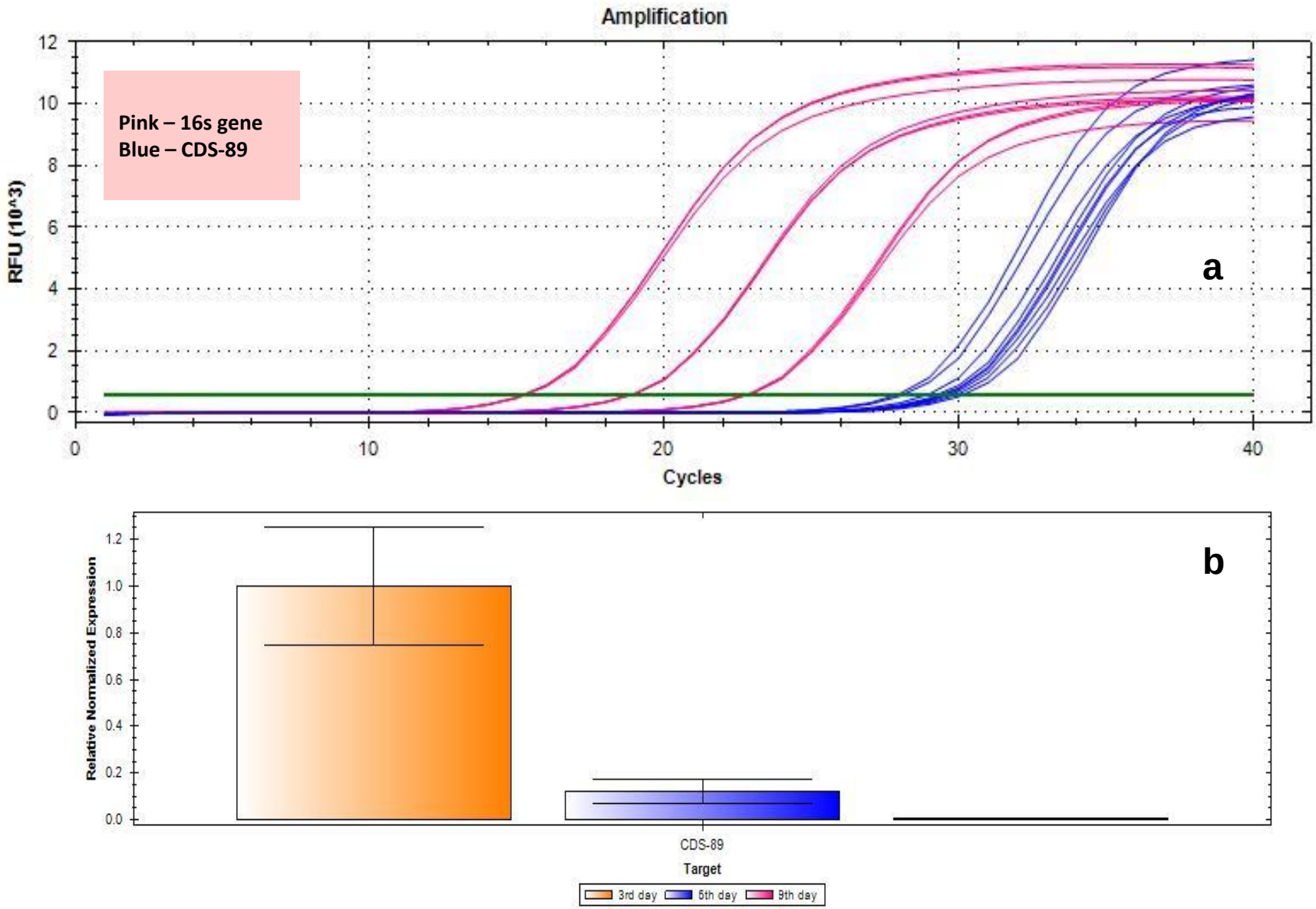
Heat map of differentially expressed genes in *Azotobacter chroococcum* (*Az*) vs. *T. viride* – *A. chroococcum* (*Tv-Az*) biofilm



Heat map of differentially expressed genes in *Trichoderma viride* (*Tv*) vs. *T. viride* – *A. chroococcum* (*Tv-Az*) biofilm

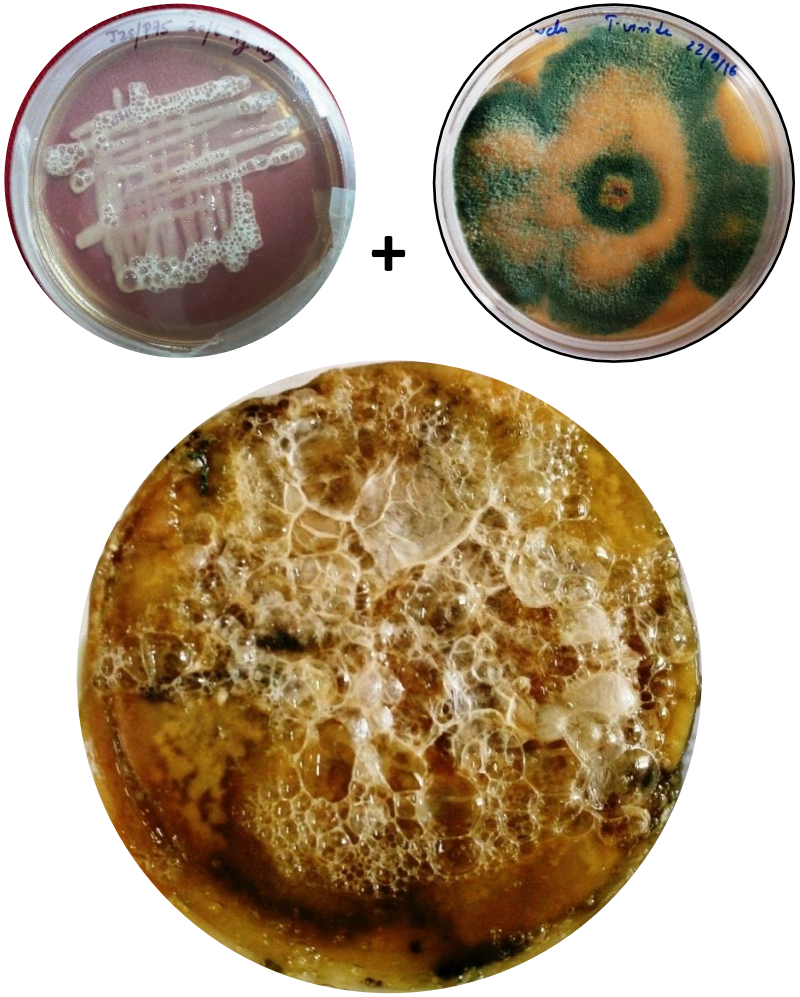


Gene expression analysis of biofilms a) Up-regulation of gene expression in CDS-366, CDS-12703, CDS-10036, CDS-5300 and CDS-13443 over control; b) relative normalised expression of up-regulated genes (CDS-366, CDS-12703, CDS-10036, CDS-5300, CDS-13443) on 3rd, 6th and 9th day of biofilm formation



Gene expression analysis of biofilms a) Down-regulation of gene expression in CDS-89 as compared to control. Relative; b)normalised expression of down-regulated gene (CDS-89) on 3rd, 6th and 9th day of biofilm formation

Conclusions and future prospects



- The dual species biofilm **interacted synergistically** in terms of its functionality, leading to enhanced growth and nutrient availability and uptake, indicative of its better survival in the crop rhizosphere

- The implications of these improvements on cotton **productivity, quality and biocontrol potential against insects and fungal/bacterial pathogens** needs to be investigated, for its potential use an environment-friendly input in cotton farming.

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Thanks