Indian Journal of Agricultural Sciences **93** (10): 1126–1131, October 2023/Article
https://doi.org/10.56093/ijas.v93i10.140808

Genetic diversity assessment and selection of *Bradyrhizobium* strains for Inceptisols based on symbiotic performance

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Received: 14 August 2023; Accepted: 23 August 2023

ABSTRACT

The symbiotic association of pigeonpea (*Cajanus cajan* (L.) Millsp.) with rhizobia plays a pivotal role in plants' growth and development. The present study was carried out at Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh during 2018–2020 to explore the diversity of rhizobia present in the *Cajanus cajan* root under Inceptisols and identify an elite strain that exhibits exceptional plant growth promotion and of nitrogenase activity for efficient nitrogen fixation. The bacterial identification using 16s rDNA sequencing revealed bacteria strains *Bradyrhizobium japonicum* (S12), *Bradyrhizobium subterraneum* (S1, S7, S8, S10, S13), *Bradyrhizobium yuanmingense* (S2, S3, S4, S6, S9, S11) while two bacteria were the endophytes identified as *Pseudomonas azotoformans* (S5) and *Paenibacillus seodonensis* (S15). Genetic diversity using UPGMA clustering revealed remarkable genetic variations, classifying 14 strains into three primary clusters and four secondary clusters. Strains S1, S3, S6, S9, and S13 exhibited elevated nitrogenase activity, indicative of their proficient nitrogen fixation capability. In plant growth experiments, all strains demonstrated significant growth of the plants compared to control. The notable growth enhancement by these five strains can be attributed to their remarkable efficiency in nitrogen fixation, as indicated by significantly higher nodule formation and nitrogenase activity. These findings provide valuable insights for identifying potential rhizobial inoculants to enhance pigeonpea productivity.

Keywords: *Bradyrhizobium*, *Cajanus cajan*, Endophytes, Genetic diversity, Nitrogen fixation

The intricate symbiotic relationship between rhizobial strains, particularly *Bradyrhizobium*, and Pigeonpea [*Cajanus cajan* (L.) Millsp.] play a crucial role in nitrogen fixation (Kumar *et al*. 2021a). Nitrogen fixation (NF) enables plants to acquire atmospheric nitrogen and convert it into a usable form, thus reducing the dependence on synthetic fertilizers and promoting sustainable farming practices (Goyal *et al*. 2021). *Cajanus cajan*, as a legume crop, exhibits compatibility with specific slow-growing strains of rhizobia, predominantly genus *Bradyrhizobium*. These rhizobia establish a symbiotic association with *Cajanus cajan* to convert atmospheric nitrogen into ammonium through the enzyme nitrogenase, which is then assimilated by the plant for various metabolic processes (Varun *et al*. 2017).

Different rhizobial strains may exhibit variations in their nitrogen-fixing abilities, adaptability to specific soil conditions, and responsiveness to environmental factors (Goyal *et al*. 2021). Therefore, understanding the diversity and identification of rhizobial strains associated with *Cajanus cajan*, particularly in the hot and humid regions of India, becomes essential for optimizing NF and improving crop production. However, despite the significance of symbiotic association between rhizobia and *Cajanus cajan*, there is limited knowledge regarding the specific rhizobial partners of *Cajanus cajan*, particularly in the context of hot and humid regions of India, the major *Cajanus cajan*-growing areas (Arora *et al*. 2018). Previous studies have primarily focused on interactions between rhizobia and related legume hosts such as peanut, cowpea and other legume crops (Goyal *et al*. 2021).

Microbial genetic diversity, pivotal for ecological understanding and applications, is effectively explored through 16s ribosomal RNA (rDNA) gene analysis (Palaniappan *et al.* 2010). This gene's conserved and variable regions facilitate phylogenetic study and differentiation of microorganisms. In this study, 16s rDNA analyses were employed to investigate genetic diversity, aiming to reveal evolutionary relationships and genetic variations, among the isolates. The objective of this study was to address the knowledge gap regarding the diversity of rhizobial isolates

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in Inceptisols and their symbiotic impact on the growth and development of *Cajanus cajan*. The findings of this research can have practical implications for facilitating sustainable agricultural practices, improving yields, and contributing to food security in the region.

MATERIALS AND METHODS

The present study was carried out at the Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University (BHU), Varanasi, Uttar Pradesh during 2018–2020 to characterize indigenous rhizobial strains nodulating pigeonpea from hot and humid regions of India.

Sample collection: Fourteen root nodule samples of *Cajanus cajan* plants (60 days old) (Degefu *et al*. 2018) growing under Inceptisols, sandy loam texture and hot and humid climatic conditions were collected (Table 1). The selection of these specific places was based on their relevance to the study objectives, considering the diversity of soil types and climatic conditions in these regions.

Isolation and purification of bacterial strains: Bacteria were isolated as described by Purwaningsih *et al*. (2019) on autoclaved Yeast Extract Mannitol (YEM) agar medium with a 1.5% agar concentration under aseptic conditions. Bacterial colonies displaying white, translucent, and elevated characteristics were carefully selected and continuous sub-culturing of these isolates was performed.

Growth kinetics in YEM medium: Bacterial strains were inoculated with 100 µL diluted bacterial suspension in three replications and incubated at 28°C on a rotary shaker set at 125 rpm. Growth was assessed by measuring the optical density at 420 nm using a spectrophotometer (Genenye 20 USA) after 2, 3 and 7 days post-inoculation (Degefu *et al.* 2018).

Genetic diversity of the bacterial isolates: Bacterial diversity was assessed by 16s rDNA gene sequences. DNA extraction followed the method outlined by Wright *et al*. 2017.

Table 1 Source of the sampled root nodules, collected for bacterial isolation and subsequent study

Strain name	Place of sample collection
S1	Begusarai, Bihar
S2	Barauni, Bihar
S3	Kushamahaut, Begusarai, Bihar
S4	Bihat, Begusarai, Bihar
S5	Mahua, Vaishali, Bihar
S6	Samastipur, Bihar
S7	Hajipur, Vaishali, Bihar
S8	BHU, Varanasi, Uttar Pradesh (UP)
S9	Mau, UP
S10	RGSC, BHU, Barkachha, UP
S11	Raja Talab, Varanasi, UP
S12	Sadaat, Gazipur, UP
S13	Jakhaniyan, Gazipur, UP
S15	Parsonpur, Mirzapur, UP

PCR amplification of 16s rDNA gene: Bacterial species were identified through 16s rDNA gene sequencing, employing the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTTGTTACGACTT-3') (Lane 1991). The amplification reaction contained 1.5 mM $MgCl_2$, 2.5 mM each dNTP, 25 pmoles of each primer, 50 mg DNA template, and 3 U of Taq DNA polymerase along with its reaction buffer. PCR cycle involved an initial denaturation at 94°C (5 min), denaturation at 94°C (45 sec), annealing at 56°C (45 sec, 30 cycles), extension at 72°C (90 sec), final extension at 72°C (10 min), and holding at 4°C. Gel visualization and documentation were performed under a UV trans-illuminator and gel documentation system, respectively. Amplicons were purified using the MinElute Gel Elution Kit (Qiagen, catalog no. 28604) and purified 16s rDNA amplicons were subjected to sequencing using the Applied Biosystems 3130 Genetic Analyzer, in collaboration with an external sequencing facility.

Analysis of 16s rDNA sequences: The resulting sequences were aligned via BioEdit software, version 7.2.5 (Hall 1999). Subsequently, a comparison was made with NCBI databases using the Nucleotide Basic Local Alignment Search Tool (BLAST) program, available at the NCBI BLAST server. The sequences exhibiting 99% similarity were identified. The determined nucleotide sequences have been submitted in the NCBI database.

Plant growth experiment: The experiment took place in a pot with sterile sand in the controlled greenhouse (30±2°C, photoperiod: 16 h) at BHU, Varanasi, Uttar Pradesh. Seedlings from sterilized seeds (Variety: MA6) were immersed in bacterial cultures for 2–3 min. Uninoculated seedlings served as the control. Seedlings were planted in the pots, with an additional inoculation of 5 ml of bacterial cultures and regular watering with Thornton media (*p*H 7.0). After 75 days, plants were harvested with minimum disruption to the roots and nodules. Data were recorded for plant height (PH), root length (RL), leaf count/plant (LCP), nodule count/plant (NCP), roots fresh weight (RFW) and shoots fresh weight (SFW), roots dry weight (RDW) and shoots dry weight (SDW), and nodules dry weight (NDW). The experimental design encompassed completely randomized design in five replications. Yield (Q/ha) was recorded under field conditions. All the treatments (inoculated) and control (uninoculated) were grown in the plot size of 8 m^2 in randomized block design in three replications. All the recommended agronomic practices were followed uniformly for all the treatments as well as control.

Nitrogenase test and N content estimation: Nitrogenase activity was assayed using acetylene reduction assay (Stewart *et al*. 1968). Nitrogen content in the shoots of 75 days old pigeonpea plants were determined by Modified Kjeldahl Method as per procedure outlined by Gupta (2012).

Statistical analysis: Statistical analysis was done for generating an Analysis of Variance (ANOVA) (Gomez and

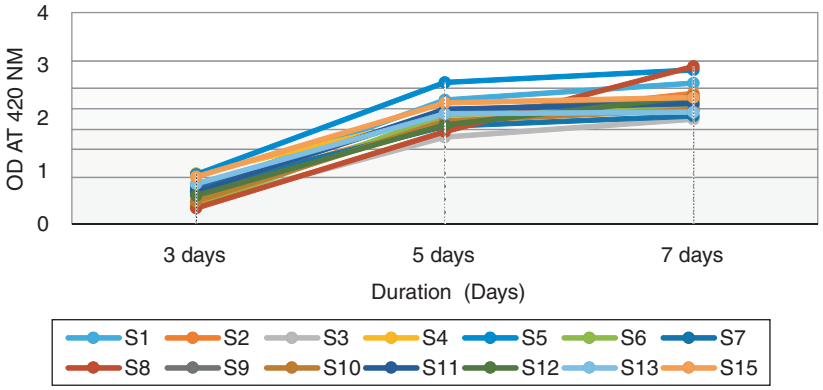

Fig 1 Bacterial growth pattern at different time intervals.

Gomez 1984). The significance among various treatments were assessed using the least significant difference at a 5% error degree of freedom. Data analysis was performed using the agricolae package in R studio (version R.4.0.2).

RESULTS AND DISCUSSION

Relative growth profile of the bacterial isolates: The growth of the bacterial isolates showed initial slow growth for the first three days (Fig 1). This initial lag phase might be due to strain adaptation to the growth medium and limited essential nutrients. Subsequently, the isolates entered the exponential growth phase, efficiently utilizing nutrients in the YEM broth and adapting to laboratory conditions (Robador *et al*. 2018). However, on the fifth day onwards, growth rate slowed down, signifying the attainment of the stationary phase. In this phase, cell division balances with cell death, and growth stabilizes due to nutrient depletion or accumulation of toxic by-products. This typical behaviour confirms the classification of the isolates as *Bradyrhizobium* strains (Degefu *et al*. 2018).

Genetic diversity of the bacterial isolates: Genetic diversity analysis revealed that the majority of the strains (12 out of 14) belonged to the genus *Bradyrhizobium*. Additionally, one strain was identified as *Pseudomonas azotoformans*, and another as *Paenibacillus seodonensis* (Table 2). The identification of the bacterial strains through molecular-based techniques, such as 16s rDNA analysis, is widely employed in microbial characterization studies (Palaniappan *et al.* 2010).

Evolutionary history of the bacterial strains through a

Table 2 Molecular characterization and identification of bacterial isolates isolated from pigeonpea

Bacterial isolate/strain	Name of the bacterial isolate	Length (bp)	Matching with NCBI database including accession number	% Similarity	Accession number
S1	*Bradyrhizobium subterraneum*	1386	*Bradyrhizobium subterraneum* strain 5252E 16s rDNA, Sequence ID: MT501100.1	100	MW547460
S2	*Bradyrhizobium yuanmingense*	1360	*Bradyrhizobium yuanmingense* strain APP20 16s rDNA, Sequence ID: MT533796.1	100	MW566702
S3	*Bradyrhizobium yuanmingense*	1350	*Bradyrhizobium yuanmingense* strain APP26 16s rDNA, Sequence ID: MT533802.1	100	MW566703
S4	*Bradyrhizobium yuanmingense*	1350	*Bradyrhizobium yuanmingense* strain APP26 16s rDNA, Sequence ID: MT533802.1	100	MW566704
S5	*Pseudomonas azotoformans*	1172	*Pseudomonas azotoformans.* strain JMAGO2_3 16s rDNA, Sequence ID: MG757959.1	99	OR016148
S6	*Bradyrhizobium yuanmingense*	1350	*Bradyrhizobium yuanmingense* strain APP26 16s rDNA, Sequence ID: MT533802.1	100	MW566705
S7	*Bradyrhizobium subterraneum*	1386	*Bradyrhizobium subterraneum* strain 5252E 16s rDNA, Sequence ID: MT501100.1	100	MW566706
S8	*Bradyrhizobium subterraneum*	1386	*Bradyrhizobium subterraneum* strain 5252E 16s rDNA, Sequence ID: MT501100.1	100	MW566707
S9	*Bradyrhizobium yuanmingense*	1370	*Bradyrhizobium yuanmingense* strain 5450E 16s rDNA, Sequence ID: MT501098.1	100	MW566708
S10	*Bradyrhizobium subterraneum*	1386	*Bradyrhizobium subterraneum* strain 5252E 16s rDNA, Sequence ID: MT501100.1	99	MW566709
S11	*Bradyrhizobium yuanmingense*	1342	*Bradyrhizobium yuanmingense* strain APP167 16s rDNA, Sequence ID: MT544597.1	100	MW566710
S12	*Bradyrhizobium japonicum*	1379	*Bradyrhizobium japonicum* strain BVVRRN67 16s rDNA, Sequence ID: MK559521.1	100	MW566711
S13	*Bradyrhizobium subterraneum*	1386	*Bradyrhizobium subterraneum* strain 5252E 16s rDNA, Sequence ID: MT501100.1	100	MW566712
S15	*Paenibacillus seodonensis*	1475	*Paenibacillus amylolyticus* strain WAB2157 16s rDNA, Sequence ID: MH169324.1	99	MW566713

Neighbour-Joining method (Fig 2) revealed the phylogenetic relationships among the strains and their evolutionary distances. Further molecular characterization using Amplified Ribosomal DNA Restriction Analysis (ARDRA) and UPGMA clustering showed significant molecular diversity among the isolates, with three major clusters and four minor clusters identified at a similarity coefficient of 0.50 (Fig 2). Strain S5 (*Pseudomonas azotoformans*) exhibited complete separation from the other strains, while strain S15 (*Paenibacillus seodonensis*) was distinct from the twelve *Bradyrhizobium* strains. The remaining *Bradyrhizobium* spp. strains clustered together in one major cluster. Within the minor clusters, several strains of *Bradyrhizobium yuanmingense* and *Bradyrhizobium subterraneum* were grouped together, while individual strains formed separate clusters. These results highlight the diversity even among strains of the same bacterial species and suggest the influence of soil and environmental conditions across the sampled areas.

The observed molecular diversity underscores the profound influence of environmental factors on bacterial populations, shaped by varying soil conditions, ecological contexts, and host interactions (Kumar *et al*. 2021b, Banjare *et al*. 2023). It can aid in targeted agricultural applications, including biofertilizer production (Singh *et al.* 2022). The phylogenetic relationships and clustering patterns highlight the diversity even within strains of the same bacterial species, driven by variations in soil conditions across sampled areas, in line with Zhang *et al.* (2008). By accounting for the nuanced genetic makeup and ecological preferences of distinct strains, researchers can design interventions that maximize the desired outcomes.

Plant growth experiment: The results of the plant growth experiment provide valuable insights into the impact of different bacterial isolates on the growth and development of *Cajanus cajan* plants. The ANOVA (Table 3) revealed a significant difference between the treatments for all the nine traits studied, indicating the presence of variation among these isolates.

Effect of Rhizobial inoculants on morphometric parameters of Cajanus cajan: PH, RL and LCP are important indicators of plant biomass and overall growth. The significantly tallest plants were observed in treatments with strains S1 and S3. The strains S13, followed by S6, S1, S11, and S14 consistently demonstrated their efficacy through higher RL. In terms of LCP, strains S3, S1, and S6 contributed to an increased LCP, leading to enhanced photosynthesis and improved plant growth. The observed increase in plant growth can be attributed to enhanced nutrient availability by solubilization, NF and phytohormones production that promote plant growth and chelate iron, benefitting both nutrient uptake and soil health (Kaur *et al*. 2022).

RFW and SFW reflect the overall biomass and vigour of the plants. Plants treated with strains S3, S1, S6, S7, and S2 exhibited significantly higher RFW compared to the other strains. Similar effects were observed for RDW too among the plants treated with strain S1, S3, S2, S6 and S7, indicating the contribution of these strains to root biomass accumulation. SFW and SDW followed a similar pattern, with strains S3, S1, S6, and S13 displaying significantly higher values compared to the control. The increase in RDW and SDW in the inoculated treatments as compared to control confirmed the earlier finding that nitrogen availability contributes significantly to the plant growth (Allito *et al*. 2021).

Increased NCP can enhance NF, leading to improved growth and development of crop plants (Mabrouk *et*

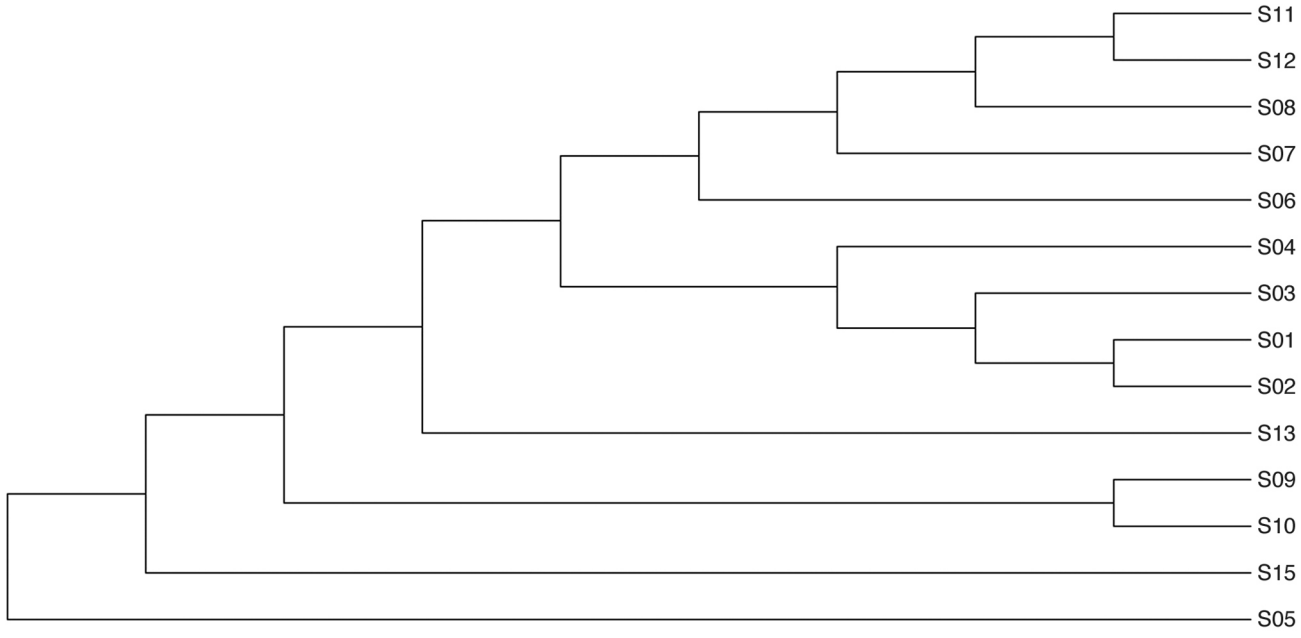

Fig 2 Phylogenetic analysis of indigenous *Cajanus cajan* bacterial isolates based on the 16s rDNA.

Table 3 ANOVA showing the variation among different strains for the different traits

	Df	PH	RL	NCP	LCP	RFW	RDW	SFW	SDW	NDW
Replication	2	7.1	1.56	0.3	3.6	0.12	0.00035	2.02	0.00102	0
Treatment	15	758.2***	56.35***	579***	319.2***	78.77***	0.0522***	70.3***	0.03347***	180.32***
Residual error	30	3	8.21	2.6	3.6	0.34	0.00049	0.49	0.00045	0.33

Significance code: '***' 0.001. PH, Plant height; RL, Root length; NCP, Nodule count/plant; LCP, Leaf count/plant; RFW, Roots fresh weight; RDW, Roots dry weight; SFW, Shoots fresh weight; SDW, Shoots dry weight; NDW, Nodules dry weight.

al. 2018). Strains S3 and S1 exhibited the highest NCP, significantly surpassing other strains. Although strains S6 and S7 exhibited a relatively lower ability to form nodules compared to S3 and S1, they still showed a significant NCP compared to the control and other strains. NDW reflects nodule biomass and NF ability. Strains S1, S3, S6, S13, and S9 exhibited significantly higher NDW compared to the other strains, indicating their ability to enhance NF. Positive correlations between NDW and nitrogenase activity have been reported in previous studies (Mendoza-Suárez *et al*. 2020). Therefore, these strains likely contribute to higher NF, resulting in enhanced growth and development of plants.

The mean yield of the plants treated with the strains S3, S13, S11 and S9 showed significantly higher yield than the plants treated with other strains. These strains likely exhibit a high level of compatibility with *Cajanus cajan*, leading to efficient nodulation and subsequent NF. The enhanced nitrogen availability likely translates into improved plant vigour, increased biomass accumulation, and ultimately, higher yield (Goyal *et al.* 2021).

This study unveils statistically significant positive correlation across multiple attributes. Notably, the utilization of efficacious isolates, specifically S1, S6 and S13, engendered a positive association between PH and RL. It suggests a functional relationship wherein increased PH corresponds to extended root systems, potentially enhancing nutrient and water uptake through augmented surface area. This observation aligns with prior research by Cochavi *et al*. (2020). This study revealed a positive correlation between nodule parameters and key attributes, including PH, RL, and biomass accumulation exerting a beneficial influence on plant growth. The presence of more NCP enhances NF, which subsequently improves nutrient availability and overall plant development (Schwember *et al.* 2019).

The observed positive correlations among diverse growth parameters elucidate a synchronized effect, wherein the augmentation of one attribute exerts a favourable impact on others. The evaluated strains, notably S1, S3, and S6, manifest favourable properties in promoting plant growth, evidenced by elevated values of PH, RL, NCP, LCP, RDW and SDW. This substantiates the potential utility of these bacterial isolates in advancing the growth and maturation of *Cajanus cajan*, thus holding promising implications for fostering sustainable crop production (Allito *et al*. 2021, Goyal *et al*. 2021).

Nitrogenase activity of the bacterial isolates and nitrogen content of 75 days old shoots: The nitrogenase activity exhibited distinctive patterns across bacterial strains, with strains S3, S6, S9, and S13 showcasing notably higher activities in comparison to the remaining strains. Remarkably, these four strains demonstrated statistically comparable levels of nitrogenase activity. This enzyme activity aligns with the outcomes of the plant growth experiment, where plants inoculated with specific strains exhibited significantly enhanced growth compared to plants treated with other strains (Goyal *et al*. 2021, Purwaningsih *et al.* 2021).

A fundamental requirement for the selection of a *Rhizobium* strain for legume inoculation is its capacity to efficiently fix nitrogen (Mendoza-Suárez 2020). The nitrogen estimation of shoots also demonstrated the higher nitrogen content in the plants treated with strains S3 and S13. All tested strains exhibited nitrogen provisioning to the plants. However, notable variations in effectiveness were observed during plant growth experiments, indicating a host-specific response.

In conclusion, this study unveils the pivotal role of rhizobial associations in enhancing the growth and development of *Cajanus cajan* under Inceptisols. The identification of potent strains S1, S3, S6, S9, and S13 underscores their exceptional NF capabilities and profound impact on plant growth. Through meticulous genetic analysis, the study provides novel insights into the diversity and clustering of these strains. These findings hold immense promise for addressing agricultural challenges, particularly in water-scarce and high-temperature regions, by harnessing the potential of these strains for sustainable pigeonpea productivity.

ACKNOWLEDGMENT

The author is grateful for JRF fellowship from CSIR.

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