



Genomic and physiological plasticity in natural variants of commercial soybean inoculants supports the non-GMO status of base-edited inoculants

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Abstract

The regulatory classification of genome-edited microorganisms remains a topic of debate, particularly in the agricultural sector. In this study, we analyze the genomic and physiological differences of commercial variants of *Bradyrhizobium japonicum* E109, a widely used soybean inoculant, to establish a reference framework for distinguishing natural genetic variation from intentional genome editing. Specifically, we conducted whole-genome sequencing and phenotypic assessments of E109 variants obtained from commercial inoculants, comparing them to the official strain maintained by INTA. Our results reveal that commercial variants naturally accumulate multiple single-nucleotide substitutions, with an average of 9.7 mutations per variant, comparable to a CRISPR base-editing event targeting at least 6 different loci. Notably, the majority of these mutations occur within coding sequences (91%), and most of them (82%) are non-synonymous, potentially affecting protein function and inoculant fitness. In vitro and *in planta* assays confirmed that these natural mutations impact key traits, including oxidative stress resistance, root attachment, cell survival, and nitrogen fixation efficiency. These findings provide a scientific basis for regulatory decisions by demonstrating that genomic variability within commercial inoculants is a natural process and support the classification of base-edited inoculants as equivalent to non-genetically modified organisms (non-GMOs).

Key message

Natural genomic and phenotypic variability in commercial soybean inoculants supports the classification of base-edited inoculants as non-GMO.

Keywords Inoculants · Natural variants · Edited bacteria · Non-GMO · Rhizobia · Soybean

Introduction

Since its emergence in the 1980 s, genetic engineering of plant has been a pivotal tool for improving crop productivity. The development of genetically modified organisms (GMOs) has enabled the introduction of traits such as herbicide tolerance and resistance to biotic and abiotic stress, significantly enhancing agricultural efficiency (ISAAA 2025). These advancements were expanding by a robust regulatory framework that ensured the safe release of genetically modified crops by evaluating the potential risks associated with foreign DNA integration.

In recent years, regulatory frameworks have adapted to integrate emerging genome-editing technologies, such as

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the CRISPR/Cas9 system, enabling the commercialization of genetically modified organisms as conventional, as long as no foreign DNA is introduced (Ayub and Soto 2023). This shift has opened new possibilities for plant growth-promoting bacteria, commonly known as bioinoculants, as direct targets for genetic engineering with tangible agro-industrial applications. Unlike traditional approaches where microorganisms played an indirect role—such as being cloned into vectors for plant or animal transformation—this new paradigm enables direct genetic modifications to enhance microbial functions in agronomy.

An increasing number of countries, including major food producers such as the United States, China, India, Brazil, Bangladesh, Canada, Australia, and Argentina, recognize genome-edited bioinoculants as conventional when no foreign DNA is introduced (GGERT 2025). However, regulatory approaches vary, with case-by-case differences reflecting diverse interpretations of how edited inoculant should be classified and regulated. A key topic of debate within the scientific and technical committees of regulatory agencies is whether a specific threshold of genetic modifications should be established to classify inoculants as traditional. If such a threshold is defined, the discussion focuses on determining a scientifically justified limit that aligns with the range of genetic variations that can occur naturally.

In this study, we contribute to the ongoing debate by analyzing the genomic and phenotypic variations of *Bradyrhizobium japonicum* E109, one of the most widely used soybean inoculants worldwide (Liebrenz et al. 2022b). We conducted a comparative analysis of the genome and both *in vitro* and *in planta* traits of the reference E109 strain from official culture collections (INTA, Argentina) and commercially available strains currently marketed as “E109”. These commercial strains, obtained from medium and large companies and hereafter referred to as “variants”, were originally supplied by INTA to these companies and may have undergone evolutionary changes over time. This report provides valuable insights into the natural evolution occurring within the ecosystem of inoculant-producing companies. By understanding these dynamics, we can better distinguish the extent of variability that arises naturally from the agro-industrial environment and, in turn, determine the scope of genomic editing that can be applied without disrupting the expected genetic diversity within these particular systems.

Methods

Commercial variants were isolated from inoculation kits or pelleted seeds obtained from seven independent inoculant companies and cultured on YEM agar plates (10.0 g/L mannitol, 0.5 g/L K_2HPO_4 , 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L

NaCl, and 1.0 g/L yeast extract, 15 g/L agar). For each company, three independent single colonies were isolated from YEM plates and sequenced, all showing identical nucleotide sequences. To identify putative mutation sites associated with the natural divergence of variants of the wild-type strain E109, genomic DNA from these inoculants and the reference strain E109 from the INTA collection (used as a control) was isolated from overnight cultures using the Wizard Genomic DNA Purification Kit (#A1120, Promega, USA). Genome sequencing was conducted at IABIMO, Argentina. A total of 3 million paired-end reads, with read lengths of 151 bp, were generated using Illumina HiSeq 1500 technology. The sequencing reads were mapped to the chromosome of strain E109 (reference sequence: NZ_CP010313) using Geneious software, as previously (Liebrenz et al. 2022b). The analysis was performed with the following parameters: Mapper = Geneious, Sensitivity= Low Sensitivity/Fastest, Fine Tuning= None (fast/read mapping), Minimum Mapping Quality= 30, and Map Multiple Best Matches= Randomly.

Tolerance to H_2O_2 was measured as previously described (Liebrenz et al. 2022b), with slight modifications. Briefly, bacterial cultures were grown to mid-log phase in YEM medium for 7 days at 30 °C with shaking at 200 rpm. Cultures were then adjusted to an OD_{600} of 1 and evenly spread onto YEM agar plates. Sterile Whatman No. 1 filter disks (5 mm diameter) impregnated with 5 μ L of 10% (w/w) H_2O_2 were placed on the surface of the bacteria-seeded plates. Inhibition zones were measured after incubation at 30 °C for 4 days. Doubling time was determined in exponentially growing cells by monitoring optical density at 580 nm (OD_{580}) in cultures grown in RMM medium under aerobic conditions at 28 °C with constant agitation (250 rpm), following previously described methods (Liebrenz et al. 2022a). To evaluate cell survival under low-nutrient conditions, YEM cultures of the variants were transferred to a physiological solution (0.9% NaCl) and incubated for two months. Survival was quantified as the percentage of colony-forming units (CFUs) relative to the initial count at time zero, which was set as 100%.

Root attachment analysis was conducted as previously described (Ledermann et al. 2018), where the presence of inoculants was assessed using YEM agar plates and by amplifying and sequencing the *hisS* gene from E109 (WP_014491987.1). Nodule occupancy was determined as previously described (Ledermann et al. 2015). In competition experiments, the presence of commercial variants and a spontaneous streptomycin-resistant (Str^R) mutant derived from strain E109 was evaluated using YEM agar plates, with and without 400 μ g/mL streptomycin. Nitrogen fixation efficiency of both wild-type and mutant strains was analyzed by inoculating soybean plants with rhizobia and growing them hydroponically in 100% vermiculite,

irrigated with nitrogen-free INTA13 minimal medium as previously described (Liebrenz et al. 2022b).

Results and discussion

The E109 strain has been available on the market since the early 1990 s and was first sequenced in 2015 (Torres et al. 2015). INTA, the official institution of Argentina responsible for its conservation, preserves it through periodic sub-culturing and storage at -70°C . Before analyzing the genomes of commercial variants derived

from the E109 strain—distributed by INTA to inoculant companies prior to 2018—we first examined the genome of E109 clones from INTA's 2019 and 2024 strain collections. As anticipated, both clones, designated R1 and R2, exhibit single-nucleotide substitutions relative to the annotated genome (Table 1). Specifically, four mutations were identified in each clone (Table 1). These results suggest that the long-term preservation process of the reference inoculant leads to the emergence of point mutations. Interestingly, only one mutation is shared between both clones (trmD-T510G, RN69_RS01746) (Table 1), indicating the presence of clonal genomic variability within

Table 1 Identified mutations in commercial variants (V1–7) and in the INTA reference clones from the 2019 (R1) and 2024 (R2) strain collections, compared to the E109 clone sequenced by INTA in 2013 (NZ_CP010313.1)

Strain	Nucleotide	AA	Locus	Strain	Nucleotide	AA	Locus
V1	G179 C	R60P	01830	V5	T510G	N170 K	02145
	T510G	N170 K	02145		G221 A	S69L	03530
	A1792G	S598P	17760		C268G	L90 V	10295
	T400 A	I134 F	18690		G1938 C	G646G	25785
	G496 C	V166L	19235		T1412 A	Y471 F	33340
	C393G	Y131*	19275		A1149 T	A383 A	33885
	C1954 T	L652 F	21985		G70 C	P24 A	34370
	G1297 A	R433 C	35420		C166 T	D56 N	34645
V2	C449G	G150 A	39275	V6	C449G	G150 A	39275
	C876G	Q292H	01560		T510G	N170 K	02145
	C1212 T	G404G	01680		T550 A	S184 T	07890
	T510G	N170 K	02145		G2,101,833 C	N/A	IR
	T1,423,891G	N/A	IR		G533 C	R178P	10290
	T158 A	D53 V	10070		T566 A	L189Q	11505
	T2,332,131G	N/A	IR		A525G	H175H	11945
	C548G	S183*	33270		G497 C	R166 T	11995
V3	C125 T	A42 V	34120	V7	T1019 A	F340Y	12260
	G633 A	P220S	37230		C764 A	A255E	12265
	T510G	N170 K	02145		G1367 C	A456G	12485
	G169 T	R57S	04845		C449G	G150 A	39275
	C421 A	S133S	38620		G118 C	G40R	44855
	T382 A	K128*	38665		T510G	N170 K	02145
	C449G	G150 A	39275		C2538G	T846 T	05535
	T236G	V79G	41190		T608 A	V203E	20370
V4	C315G	P105P	44510	R1	T929 A	V310D	20975
	T510G	N170 K	02145		C1329 T	R443R	21505
	T1076 A	M359 K	03790		T214 A	I72 F	35005
	G1119 A	ExE	03790		C1320G	Q440H	35140
	G1275 C	PxP	03790		C8,193,300 A	N/A	IR
	T547 A	I183 F	19485		C449G	G150 A	39,275
	T398G	Q128P	20460		T510G	N170 K	02145
	A389 T	K130M	21590		A8,192,126 C	N/A	IR
V4	C870G	I290M	21985	R2	C449G	G150 A	39275
	A8,192,688 C	N/A	IR		A9,166,945G	N/A	IR
	A4578G	H1526H	38955		T510G	N170 K	02145
	C449G	G150 A	39275		T404 A	F135Y	05500
	A9,166,945G	N/A	IR		A4578G	H1526H	38955
					C449G	G150 A	39275

The column named “Locus” displays the locus number without the RN69_RS prefix

ND: not detected. N/A: Not applicable. IR: intergenic region

the strain collections. These results align with previous studies on the identification of spontaneous mutants derived from the E109 strain, which have demonstrated beneficial effects. In these studies, detecting such beneficial mutations required sequencing the E109 subculture used directly for mutant selection as a control, which consistently exhibited at least two point mutations compared to the annotated genome (Brambilla et al. 2023; Liebrezn et al. 2022a, b).

The analysis of the genomes of commercial variants (V1–7) shows that each variant carries between 7 and 13

single-nucleotide mutations, with an average of more than 9.7 mutations per variant (Table 1). Similar to what was observed in the control clones R1 and R2, these variants share only one common mutation (trmD-T510G) (Table 1). These findings suggest that, within the agro-industrial ecosystem, it is natural for the inoculants in use to differ from the reference inoculants provided by official institutions, containing at least 6 additional single-nucleotide substitutions in different genomic locations. These modifications would be equivalent to a CRISPR/Cas9 multiplex editing event with base-editing guides targeting 6 different loci.

It is important to note that genomic sequencing was performed using short-read technology (Illumina), which does not allow the identification of macroevolutionary events such as chromosomal rearrangements, large duplications and transpositions of mobile elements. However, such events may have occurred and could be detected in the future using long-read sequencing technologies (e.g., PacBio or Oxford Nanopore's MinION), providing a more comprehensive understanding of the natural variability associated with these evolutionary processes within the agro-industrial ecosystem.

If all mutations occur with equal probability, transversions would be expected to account for approximately 75% of mutations, and in the genome of strain E109, where the total length is 9,224,219 bp and coding sequences (CDS) comprise 7,933,279 bp, around 86% of mutations would be expected to fall within CDS regions. Closely aligning with these expectations, our analysis of mutations in the total set of variants revealed that, on average, 79% were transversions (Fig. 1a), and 91% were located within coding regions (Fig. 1b), as detailed in Table 1. These results align with previous analyses of spontaneous mutants from strain E109, where transversions were predominant, and most mutations occurred in CDS regions (Brambilla et al. 2023; Liebrezn et al. 2022a, b). Moreover, most mutations in CDS (82%) are non-synonymous substitutions (Fig. 1c), including premature stop codons and amino acid changes (Table 1), which can directly affect bacterial fitness inside and outside the plant host. These disruptive modifications can be achieved using CRISPR/Cas9 for gene knockouts and base editing to inactivate or enhance protein activity, respectively.

To determine the potential impact of the mutations present in the commercial variants, we conducted *in vitro* studies, including tolerance to H₂O₂ (Fig. 2a), cell survival (Fig. 2b), and doubling time (Fig. 2c), as well as *in planta* assays evaluating root attachment (Fig. 3a), nodule occupancy (Fig. 3b), nodule biomass (Fig. 3c), and plant productivity (Fig. 3d). Compared to the R1 and R2 control strains, all commercial variants (V1–V7), except for V5, exhibited differences in fitness *in vitro* (Fig. 2), *in planta* (Fig. 3), or both, in at least one trait under the laboratory

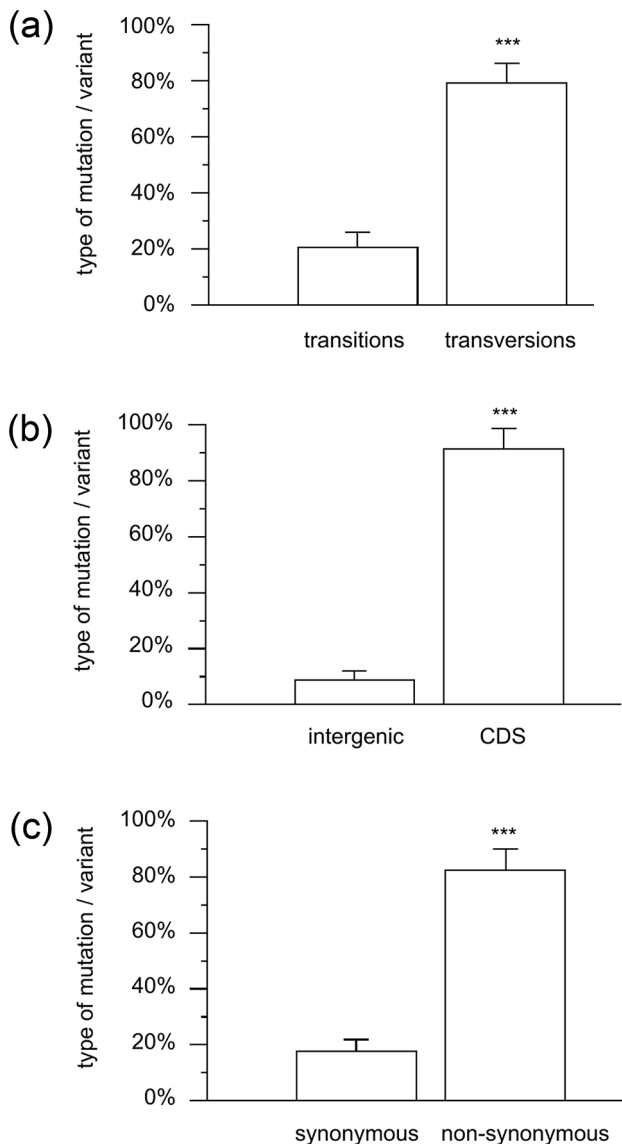


Fig. 1 Distribution of mutation types in commercial variants. The proportion of transitions and transversions (a), the proportion of mutations in coding sequences (CDS) and intergenic regions (b), and the proportion of mutations classified as synonymous or non-synonymous (c) in commercial variants, based on Table 1. Data are presented as mean $\pm$ SEM (n = 7). Statistical significance was assessed using Student's t-test (***) $p < 0.001$

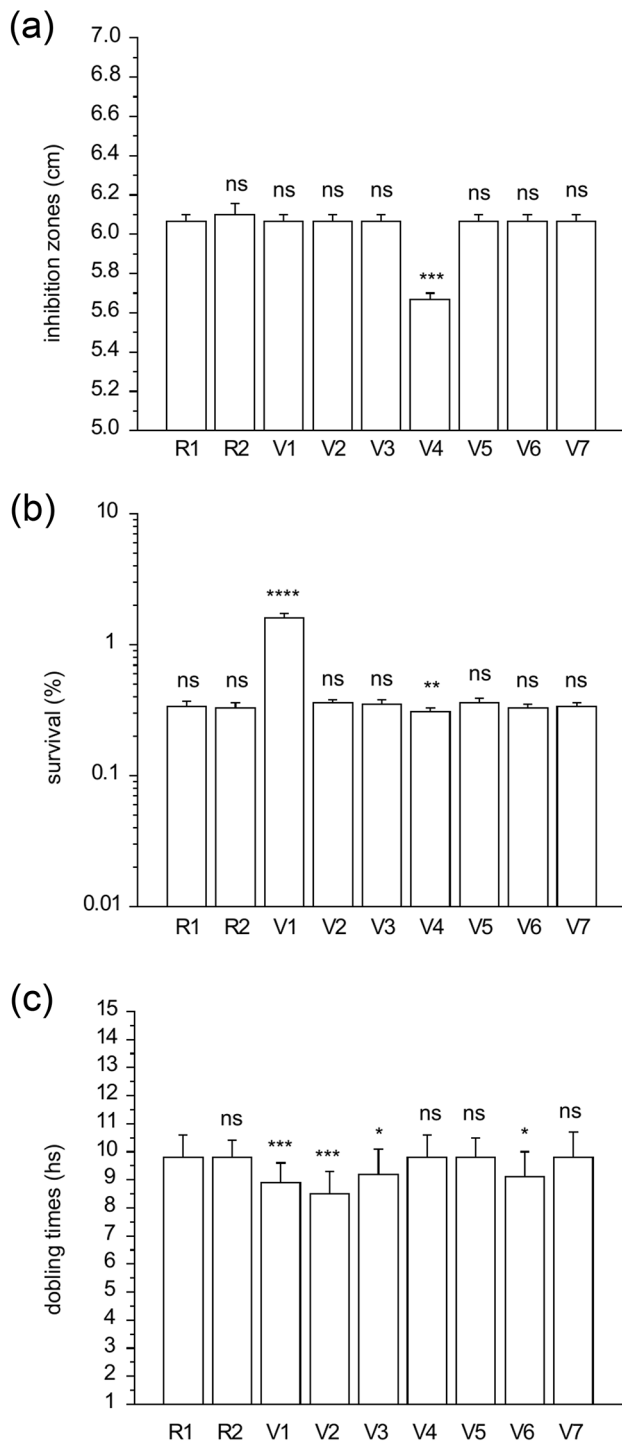


Fig. 2 In vitro characterization of commercial variants. Assessment of tolerance to **a** H_2O_2 stress, **b** cell survival under low-nutrient conditions, and **c** doubling time under optimal growth conditions in commercial variants (V1–V7) and official control strains (R1 and R2). Significant differences between inoculants were analyzed with ANOVA followed by Dunnett's multiple comparisons test (ns.: not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Values represent mean $\pm$ SEM (n = 3–10)

and greenhouse conditions tested. The most improved trait was in vitro growth rate, observed in more than half of the variants, a process that could be favored during laboratory domestication (Fig. 2c). Among the variants with the greatest functional divergence, V4 exhibited increased tolerance to H_2O_2 (Fig. 2a) and enhanced root colonization ability (Fig. 3a), traits that are typically correlated. Meanwhile, V3 displayed reduced productivity in nitrogen-free synthetic medium (Fig. 3d). These impacts could probably be attributed to synonymous substitutions in catalase (RN69_RS03790) and a nonsense mutation in a nitrogenase component (RN69_RS38665), respectively (Table 1). Regardless of the genes and modified traits in commercial variants, it is clear that spontaneous mutations naturally arising within the agro-industrial inoculant ecosystem influence their behavior, either enhancing or diminishing intrinsic abilities in a way comparable to directed evolution through genetic editing.

The challenge of containing microbial dispersion in field conditions, and consequently, the difficulty of realistically assessing the food and ecological safety of recombinant microorganisms, has significantly constrained the development of genetically modified inoculants and probiotics capable of drastically reducing nitrous oxide, carbon dioxide, and methane emissions in agriculture and livestock (Brambilla et al. 2020). However, the advent of gene editing, along with specific regulatory frameworks for these new breeding techniques, has introduced a transformative opportunity: the ability to rapidly develop genetically improved microorganisms without the net introduction of foreign DNA. This breakthrough offers new perspectives in the fight against global climate change.

For these advances to be effectively implemented at scale, regulatory frameworks must continuously evolve based on scientific evidence, ensuring that edited microorganisms are equivalent to their naturally occurring counterparts. In this regard, understanding that commercial variants exhibit differences from official reference strains, and that these variations influence microbial behavior, provides reassurance that such equivalence is genuine. Moreover, it offers concrete insights for decision-making, such as establishing that in soybean rhizobia, the presence of at least six point mutations represents a natural condition, remaining within the boundaries of natural variation. Furthermore, both in vitro and *in planta* evolutionary changes in key traits are not only expected but, according to our results, the absence of fitness variations among different variants would be the true anomaly rather than a natural state.

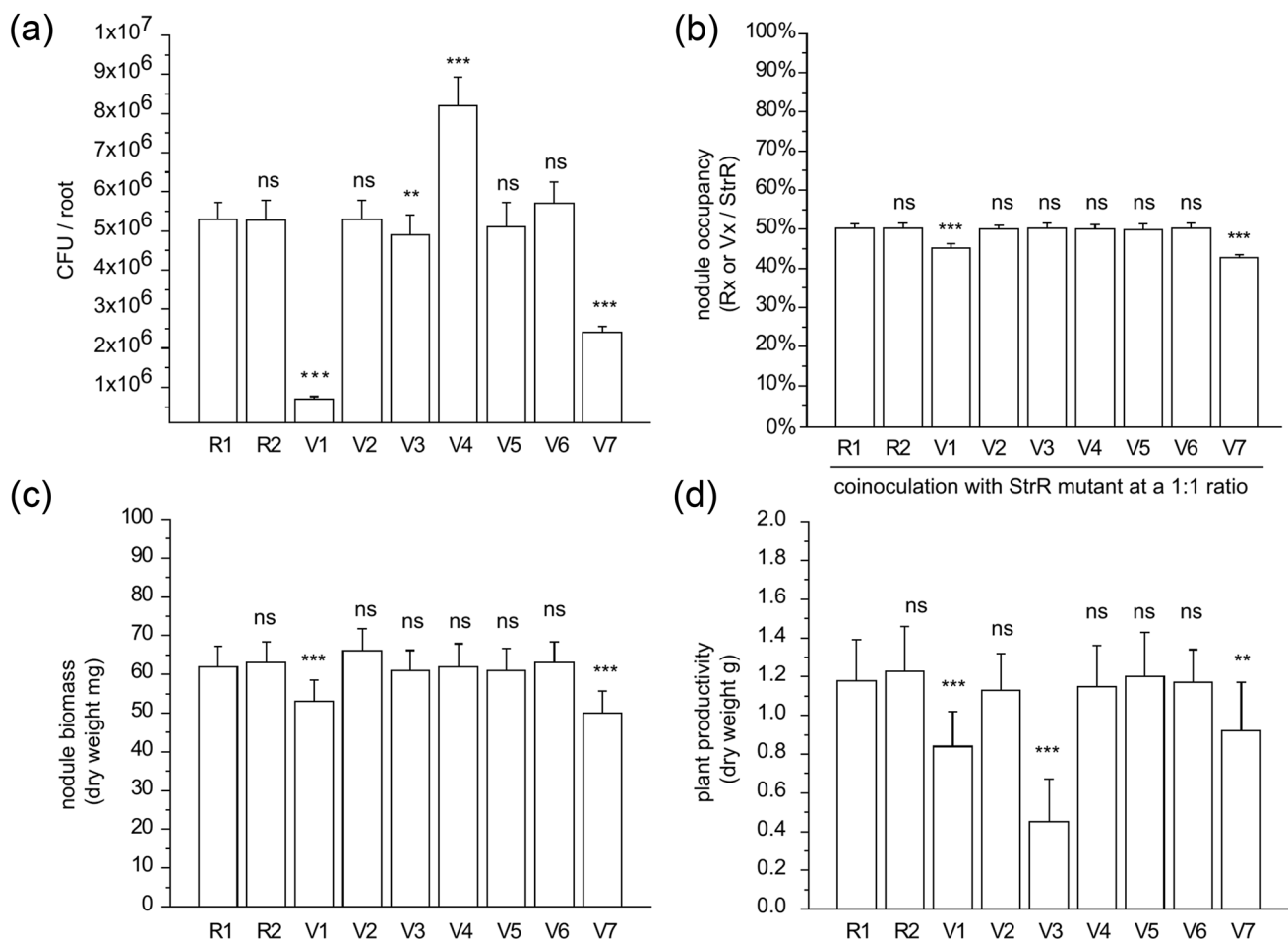


Fig. 3 *In planta* evaluation of commercial variants. Analysis of root attachment (a), nodule occupancy (b), nodule biomass (c), and plant productivity (d) in commercial variants (V1–V7) and official control strains (R1 and R2). Significant differences between inoculants were

analyzed with ANOVA followed by Dunnett's multiple comparisons test (ns.: not significant, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Values represent mean $\pm$ SEM ($n = 8$ –20)

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Data availability Data will be made available on request.

Declarations

Competing interests The authors declare that they have no conflict of interest.

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