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1 # Using variation in arbuscular mycorrhizal fungi to drive the productivity of the
2 food security crop cassava
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5 **Authors: Isabel Ceballos*, Ivan D. Mateus*, Ricardo Peña*, Diego Camilo Peña-Quemba, Chanz**

6 **Robbins, Yuli M. Ordoñez, Pawel Rosikiewicz, Edward C. Rojas, Moses Thuita, Deusdedit Peter**

7 **Mlay, Cargele Masso, Bernard Vanlauwe, Alia Rodriguez¶, Ian R. Sanders¶**

8 * These authors contributed equally to this study

9 ¶ Joint senior authors

10
11 **Corresponding author contact: ian.sanders@unil.ch**
12 **Tel: +41 79 536 7546**
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15 **The unprecedented challenge to feed the rapidly growing human population can only be**
16 **achieved with major changes in how we combine technology with agronomy[1]. Despite their**
17 **potential few beneficial microbes have truly been demonstrated to significantly increase**
18 **productivity of globally important crops in real farming conditions[2,3]. The way microbes are**
19 **employed has largely ignored the successes of crop breeding where naturally occurring**
20 **intraspecific variation of plants has been used to increase yields. Doing this with microbes**
21 **requires establishing a link between variation in the microbes and quantitative traits of crop**
22 **growth along with a clear demonstration that intraspecific microbial variation can potentially**
23 **lead to large differences in crop productivity in real farming conditions. Arbuscular mycorrhizal**
24 **fungi (AMF), form symbioses with globally important crops and show great potential to improve**
25 **crop yields[2]. Here we demonstrate the first link between patterns of genome-wide intraspecific**
26 **AMF variation and productivity of the globally important food crop cassava. Cassava, one of the**
27 **most important food security crops, feeds approximately 800 million people daily[4]. In**
28 **subsequent field trials, inoculation with genetically different isolates of the AMF *Rhizophagus***
29 ***irregularis* altered cassava root productivity by up to 1.46-fold in conventional cultivation in**
30 **Colombia. In independent field trials in Colombia, Kenya and Tanzania, clonal sibling progeny**
31 **of homokaryon and dikaryon parental AMF enormously altered cassava root productivity by up**
32 **to 3 kg per plant and up to a 3.69-fold productivity difference. Siblings were clonal and, thus,**
33 **qualitatively genetically identical. Heterokaryon siblings can vary quantitatively but monokaryon**
34 **siblings are identical. Very large among-AMF sibling effects were observed at each location**
35 **although which sibling AMF was most effective depended strongly on location and cassava**
36 **variety. We demonstrate the enormous potential of genetic, and possibly epigenetic variation, in**
37 **AMF to greatly alter productivity of a globally important crop that should not be ignored. A**
38 **microbial improvement program to accelerate crop yield increases over that possible by plant**
39 **breeding or GMO technology alone is feasible. However, such a paradigm shift can only be**
40 **realised if researchers address how plant genetics and local environments affect mycorrhizal**
41 **responsiveness of crops to predict which fungal variant will be effective in a given location.**

42 For millennia farmers have improved crops using naturally occurring intraspecific plant genetic variation
43 to improve productivity. However, rates of yield increase attributed to plant breeding and GMO-crop
44 technology are not considered sufficient to feed the projected global human population[1]. Beneficial soil

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45 microbes can potentially improve plant growth, but in sharp contrast to plant breeding, there has been
46 little attempt to improve them. Innovative approaches to microbial management of symbiotic
47 microorganisms could bring great benefits[5,6]. For decades, AMF have been known to increase plant
48 growth[7], although they are not consistently used in agriculture or the focus of an improvement program
49 (Supplementary information note 1).

50 We investigated whether naturally occurring variation of a commonly occurring AMF species of
51 agricultural soils (*Rhizophagus irregularis*) can significantly alter the productivity of the globally important
52 food security crop cassava. Cassava feeds almost 800 million people in 105 countries and responds
53 positively to AMF inoculation in farming conditions[4,8].

54 Establishing a link between discernible patterns of genetic variation within a beneficial microbial species
55 and plant growth has never been attempted, but if the link exists then patterns of microbial genetic
56 variation can be used as a predictor for selection of beneficial strains. In AMF, mapping quantitative trait
57 loci and genome-wide association studies are not currently realistic because of lack of recombinant
58 lines, inadequate plant phenotypic datasets, and a limited panel of isolates. Another way to identify the
59 existence of such a link is to demonstrate whether quantitative traits of a crop species are influenced by
60 the genetic relationships among individuals of an AMF species, as discerned by phylogenetic
61 relationships[9]. First, quantitative fungal growth traits were measured in a set of *R. irregularis* isolates,
62 spanning the phylogeny of this species and using data on thousands of single nucleotide polymorphisms
63 (SNPs) distributed widely across their genomes[10,11]. Second, we inoculated one cassava cultivar (NGA
64 16) with a set of 11 *R. irregularis* isolates also spanning the *R. irregularis* phylogeny. Indeed, the fungal
65 isolates differed significantly in their quantitative growth traits (Supplementary information table S1) and
66 there was a significant phylogenetic signal on spore density and clustering (Supplementary figure 1;
67 Supplementary information table S1). The fungal isolates differentially colonized the roots and had a
68 strong and significant differential effect on cassava growth, with the strongest effect on cassava root dry
69 weight (Supplementary information table S1). Patterns of genome-wide variation among the isolates
70 were significantly associated with root dry weight and total cassava dry weight (Figure 1; Supplementary
71 information table S1). This establishes the first clear a link between genetic signatures of a beneficial
72 microorganism and growth of a globally important crop plant.

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73 Cassava has previously been shown to benefit from inoculation with *R. irregularis* under conventional
74 cassava cultivation conditions in Colombia[8,12] and represents a good candidate for an improvement
75 program (Supplementary information note 1). While AMF isolates can affect plant growth in sterile soil,
76 this is rarely validated in normal farming conditions. In a third experiment, we quantified how much
77 variation in the growth of cassava (cultivar CM4574) occurred in normal farming conditions in Colombia
78 using 5 genetically different *R. irregularis* isolates[10]. Cassava root weight ranged from 4.0 – 5.9 kg plant^{-}
79 ^{1} among inoculated plants, with non-inoculated plants falling in the middle of this range (Figure 2;
80 Supplementary information table S2 and note 2). This translates to a projected yield alteration of up to
81 18.6 tons ha^{-1} fresh root weight between treatments inoculated with genetically different isolates
82 (Supplementary information note 3). Because isolates have been *in vitro* cultured for 16 years in an
83 identical environment, results imply genetic variation (and possible additional epigenetic variation) in
84 AMF leads to crop production differences (Supplementary information note 4).

85 Clonally produced single spore progeny of an *R. irregularis* isolate strongly altered the growth of rice in
86 sterile conditions (Supplementary information note 5)[13]. A fourth experiment tested the effect of single
87 spore progeny of two parental *R. irregularis* isolates on cassava root productivity in conventional
88 cassava cultivation conditions in Colombia (Supplementary information note 5 and table S3). The same
89 experiment was conducted in successive years, using two varieties of cassava. Cassava root fresh and
90 dry weight was differentially affected by sibling fungi but how they responded differed between cassava
91 varieties, with one variety (CM4574) responding more strongly to the fungal siblings (Supplementary
92 figure 2; Supplementary information table S4). Results from the successive trials showed that the fungal
93 treatments substantially affected cassava root weight under normal cassava cultivation in Colombia in
94 a reproducible way over time, even when the non-inoculated control was removed from the analysis
95 (Supplementary information table S4). The fundamental question of this experiment was to test whether
96 variation among progeny of parental *R. irregularis* isolates significantly altered cassava root productivity
97 and to measure the potential amplitude of the effect. Because the two parental isolates are genetically
98 different from each other, analyses across all treatments is not appropriate to answer this question. Due
99 to a strong AMF inoculation treatment by cassava variety interaction, we compared cassava root weight
100 among plants inoculated with a given parental isolate and its progeny and by cassava variety separately.
101 Root fresh and dry weight significantly differed by as much as 2.44-fold in cultivar CM4574 among plants
102 inoculated with the parental isolate C2 and its progeny (Figure 3a-d; Supplementary information table

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103 S5). Inoculation benefit, a measure of the response to inoculation compared to non-inoculated plants,
104 also differed significantly among the plants inoculated with progeny of C2 (Supplementary figure 3;
105 Supplementary information table S5). Cassava inoculated with parental isolate C3 and its progeny
106 showed a remarkable 2.94-fold difference in root weight, as well as differences in inoculation benefit in
107 CM4574 (Figure 3e-h; Supplementary figure 3; Supplementary information table S5). AMF colonization
108 of roots occurred in all treatments (Supplementary figure 2) and averaged over the two experiments,
109 neither AMF treatments nor cassava cultivar significantly influenced mycorrhizal colonization
110 (Supplementary information table S4). However, analysis of colonization in the 1st trial revealed that
111 colonization was significantly affected by inoculation treatments in cultivar CM4574 (data not shown).

112 More recently, parental isolates, C2 and C3, were shown to be homokaryon and heterokaryon
113 (containing a population of two genetically distinct nuclei), respectively[14-16]. *In vitro*-produced
114 homokaryon siblings cannot be genetically different from each other. Siblings of C3 are qualitatively
115 genetically identical, containing the same alleles (Supplementary information note 5), but can display
116 significant quantitative genetic differences in allele frequencies[15]. Variation in cassava growth might be
117 explained by epigenetic differences among homokaryon siblings and a combination of quantitative
118 genetic, plus epigenetic, differences among heterokaryon progeny. In order to quantify the amplitude of
119 the effects of fungal variation among homokaryon progeny and among heterokaryon progeny on
120 cassava productivity in the field, we conducted a fifth experiment in Kenya (one location) and Tanzania
121 (two locations). A local cassava variety and an improved variety (for tolerance to resistance) were
122 inoculated with five parental *R. irregularis* isolates and their single spore progeny in a randomised
123 complete block design (Supplementary information table S3). Two of the parental isolates (C3 and A5)
124 were heterokaryons (containing a population of two genetically distinct nuclei) and the three isolates
125 (A1, B12 and C2) were homokaryons. Effects of inoculation treatments were not consistent across
126 locations (Supplementary information table S6) or between two cassava varieties within a location
127 (Supplementary information table S7). Our goal was to quantify among-AMF sibling effects on cassava
128 growth. Thus, the analysis was separated by location, cassava variety and sibling groups. Thirty-two
129 independent tests compared effects of a given parental isolate and its siblings on cassava root weight
130 across all locations and with all cassava varieties (summarised in Figure 4; Supplementary figures 4-7;
131 Supplementary information table S8). Following correction for multiple testing, 19 tests revealed a
132 significant difference in cassava root fresh weight among plants inoculated with a given parental line

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133 and its clonal progeny. Exceptionally large differences in cassava root productivity, due to inoculation
134 with clonally produced siblings, were observed, with up to a 3 kg plant^{-1} difference in mean root fresh
135 weight (Figure 4). In many cases, at least one of the progeny induced significantly larger cassava root
136 growth than the original parent. Within sites and cassava varieties, the amount of variation in cassava
137 root productivity was similar among siblings of homokaryon parents and siblings of heterokaryon parents
138 (Supplementary information table S9; Supplementary figure 8) indicating that quantitative genetic
139 differences among AMF siblings are unlikely to contribute additional variation to cassava productivity.
140 Significant differences in fungal colonization were also observed among plants inoculated with sibling
141 fungi (Supplementary information tables S10-S11) but such effects were clearly not correlated with
142 cassava productivity. Responses to inoculation with *R. irregularis* isolates in Africa and Colombia were
143 highly cassava-variety dependent, showing that the genetics of the plant plays a strong role in
144 responsiveness. This is consistent with recent studies on variation in transcriptional responses of
145 cassava varieties to inoculation[17]. Interestingly, in this study the direction of the productivity response
146 compared to no inoculation (known as mycorrhizal responsiveness) was extremely consistent within
147 local cassava varieties but highly inconsistent in improved varieties (Supplementary figure 9), indicating
148 that plant breeding may disturb cassava mycorrhizal responsiveness.

149 This study represents several milestones. The study represents the first established link between
150 specific patterns of genome-wide variation in a beneficial microbial species and a globally important
151 crop species. The possibility of identifying variation responsible for increased cassava productivity, thus,
152 opens the door to the first genetic selection program of a beneficial microorganism. A second, and more
153 surprising milestone, is that variation among clonal offspring of an AMF parent led to additional,
154 exceptionally large significant differences in cassava growth in real-life farming conditions in multiple
155 locations. To put the result in context, a difference of 3kg plant^{-1} root weight would give a projected yield
156 difference of 30 tons ha^{-1} and yet he average global yield of cassava is only 12.8 tons ha^{-1}. Furthermore,
157 the way we cultivated single-spore clonal progeny of AMF sometimes led to a more growth-promoting
158 inoculant than the parental fungus showing that improving inoculants is a realistic possibility. There are
159 several other reasons why these results are very unexpected (Supplementary information note 6). While
160 The fact that cassava responsiveness to AMF is very strongly dependent on intra-specific AMF variation,
161 the genetic identity of cassava, as well as location, highlights the extreme naivety in current use of
162 beneficial microorganisms, such as AMF (Supplementary information note 6). We feel the current

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163 practice of using beneficial microbes to positively influence crop growth is insufficient to address the fact
164 that use of one inoculum in one location or variety may have a positive effect, while the same "beneficial"
165 microbe may have detrimental effects in another soil or plant variety. These data attest to the very strong
166 potential gains of using AMF variation to improve cassava productivity. Understanding and predictably
167 employing mycorrhizal responsiveness as a component to improve crop yield is a multi-disciplinary
168 problem requiring expertise from microbiologists, plant breeders and agronomists alike. Future
169 implementation of AMF in crops must consider variation in the microorganisms themselves, their
170 effectiveness on different plant varieties and localized soil conditions and soil microbiota.

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172 **Methods**

173 **Fungal material used in the experiments.** All *R. irregularis* isolates used in this study were originally
174 isolated from a field in Tänikon, Switzerland. Each isolate was initiated as a single spore culture and put
175 into *in vitro* culture with Ri-T transformed carrot roots in 2000 and subsequently maintained in the same
176 environment and successively sub-cultured every 3-4 months in the same media[18]. The *in vitro* isolates
177 and their origin were previously described[19]. Unless stated, all sub-culturing was conducted as in Koch
178 et al. (2004) where colonized roots with hyphae and many spores were transferred from a 3-4 month
179 old culture onto a Petri plate with fresh media[19]. Single spore cultures of each parental isolate were
180 made by transferring a single spore from a 3-4 month old culture of a parental isolate onto a new plate
181 with fresh media and a non-colonised carrot root, as described in Angelard et al. (2010)[13]. From this
182 point on, single spore cultures were maintained by sub-culturing in the same way as parental isolates
183 so that large amounts of clonally-grown spores could be produced from each single spore culture.

184 **Experiments 1 & 2: The test for a significant relationship between patterns of genetic variation**
185 **in AMF and quantitative traits of AMF and plants**

186 **Fungal material and measurements:** We cultivated *R. irregularis* isolates (A4, A5, B3, B4, B10, B15,
187 C1, C2, C4, D1 & D4) *in vitro* in split plates and characterised fungal growth traits[13]. In experiment 1,
188 there were 5 replicate petri plates of each isolate. We measured spore production of the isolates after 6
189 months of growth. To do this, we took photographs of 6 areas of $2cm^2$ in the fungal compartment of
190 each plate using a Leica stereoscope (MZ125). An automated measurement of spore number in a given
191 area was then made on each image with the open source software ImageJ (https://imagej.nih.gov/ij/).
192 In addition, we measured whether the spores were more clustered together or if they were distributed
193 more regularly, using the R package Spatstat[20]. To do this, we measured the spatial arrangement of the
194 spores produced each fungus by measuring the nearest distance to the spores from random points
195 chosen within each image. We also measured the hyphae produced by counting the number of hyphae
196 that crossed two transects of 1.44 cm length. We took 5 independent photos in each dish to make this
197 measurement.

198 **Plant material and growth conditions:** We propagated the cassava (*Manihot esculenta* Crantz) variety
199 NGA-16 (CIAT, Colombia) clonally *in vitro*. Plantlets were grown in a culture chamber (25°c, 14 hours

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200 light, 90% RH) on MS medium for 1 month. Then, we placed the seedlings in an steam sterilized (180°
201 25-min) soil substrate (Klassman seedling substrate:perlite 1:1). After 1 month of growth *ex vitro*, we
202 transferred the plantlets to the final steam sterilised (120°C for 40 min 2x) substrate (Klassman substrate
203 4:sand:clay:perlite 4:2:1:1). At this time, the plants were kept in the greenhouse at 28°C, 16 hours light,
204 70% RH. We inoculated each plant with 300 spores of the *R. irregularis* isolates (A3, B4, B10, C1, C2,
205 C3, C4, C5, D1, D4 or G1). There were 15 replicates per inoculation treatment and the plants were
206 arranged in a randomized block design. Plants were harvested after 8 months of growth. We measured
207 the height and dry weight of aboveground and belowground parts of the plants. Dry weight was obtained
208 after the plants were dried at 72°c for 6 days. Root colonization by AMF was determined by the grid line
209 intersection method after clearing roots with 10% KOH for 4 hours, acidified with HCl (1%) during 5
210 minutes and staining with trypan blue (0.10% in a lactic acid-glycerol solution) overnight[21].

211 **Genetic relatedness among *R. irregularis* isolates:** We used published ddRAD-seq data of the *R.*
212 *irregularis* isolates to estimate the genetic relatedness among isolates based on single nucleotide
213 polymorphisms[10,11]. We called SNPs following the method described in Wyss et al. 2016[10]. We then
214 produced a matrix of presence and absence of SNPs and calculated a genetic distance matrix using the
215 maximum distance between each comparison (supremum norm). Then we performed a hierarchical
216 clustering analysis to infer the genetic relationship among the *R. irregularis* isolates.

217 **Statistical analysis:** The fungal variables spore production and extra-radical mycelial density were
218 analyzed with a generalized mixed model using the Poisson family as a link function and the block effect
219 was defined as random. The spore clustering, was analyzed using a non-parametric Kruskal-Wallis test.
220 Fungal colonization was analyzed with a generalized mixed model using the binomial family as link
221 function, we defined the block effect as random. We used a mixed linear model to analyze the plant
222 variables: plant height, shoot dry-weight, root dry-weight and total dry-weight. The block effect was
223 defined as a random factor in the model.

224 **Phylogenetic signal:** We used the R package 'phylosignal' in order to test whether there was a
225 significant phylogenetic signal in fungal and plant quantitative traits and genome-wide genetic variation
226 in the fungi[9]. We calculated two different phylogenetic signal metrics; Abouheif's C mean and Moran's
227 I[22,23]. These two methods are not based on an evolutionary model and use an autocorrelation
228 approach[24].

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229 **Experiment 3: A test of whether genetically different *R. irregularis* isolates differentially affect**
230 **cassava growth under conventional farming conditions in Colombia**

231 By conventional farming conditions, we mean that the cassava crop was cultivated exactly as farmers
232 would normally cultivate cassava in this region with the same planting density, fertilization, harvesting
233 time etc. and the only difference was that some plants were inoculated with AMF. In other words, the
234 farmer did not have to modify the crop management in any way to allow inoculation. This is the case for
235 experiments 3, 4 and 5 described below.

236 The experiment was conducted in Tauramena municipality, Casanare, Colombia (72°34'23"W,
237 4°57'32"N at 219 metres above sea level). The climate is tropical with average temperatures of 18°C
238 (night) to 28°C (day), with an average air humidity of 75% and total annual precipitation of 2335 mm
239 with 172 rain days. The soil type is an Inceptisol. Cassava (cultivar CM4574; known locally as *Cubana*)
240 was planted as stem cuttings at a density of 10000 plants ha^{-1}. There were seven different inoculation
241 treatments where cassava plants were inoculated with one of the isolates A1, A5, B12, C2, C3 or
242 inoculated with the inoculum carrier without fungus, or not inoculated. The experiment was set up as a
243 randomized block design, with 6 blocks and with one replicate of each treatment per block. Each
244 treatment in each block, was represented by nine cassava plants arranged in a plot (3 x 3 plants). Each
245 set of 9 plants of one treatment were surrounded by a row of uninoculated plants so that any two plants
246 at the edge of the plots of different inoculation treatments were separated from each other by two non-
247 inoculated plants. The fungal inoculum was upscaled in an *in vitro* culture system by Symbiom s.r.o.
248 (Lanskroun, Czech Republic) and mixed with a carrier (calcified diatomite). Stems of cassava were
249 inoculated with 1g of the carrier, containing 1000 *R. irregularis* spores, which was placed around the
250 stem of the cassava at planting. Plants were fertilized in total with 100 Kg ha^{-1} urea, 100 Kg ha^{-1} di-
251 ammonium phosphates (DAP), 106 Kg ha^{-1} potassium chloride (KCl). Fifty percent of this was applied
252 at 43 days after planting date and the other 50% at 61 days after planting date. There was no artificial
253 irrigation and conventional crop management for the region was applied depending on pests, diseases
254 and weed incidence. The bulbous roots were harvested 321 days after planting after planting and the
255 root weight fresh weight per plant was measured. Values of the nine plants per treatment per plot were
256 pooled and the mean value per plot was used for further statistical analysis. We tested for a significant
257 difference in cassava root fresh weight among treatments by performing an analysis of variance using

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258 the JMP® statistical discovery software (Statistical Analysis Systems Institute, version 13). Comparison
259 of least square means differences was done using Student´s t test at 90% of significance.

260 **Experiment 4: A test of whether clonally-produced single spore siblings of *R. irregularis* isolates**
261 **C2 and C3 differentially affect cassava growth under conventional farming conditions in**
262 **Colombia**

263 **Experimental design:** The field experiments were established at the Utopia campus of La Salle
264 University (72° 179 4899 W, 5° 199 3199 N) near Yopal in Casanare, Colombia with the same
265 experiment conducted once, and then repeated in an adjacent field, in the following year. The climate
266 is very similar to that at the location of Experiment 3. Cassava varieties used in these experiments were
267 cultivar MCOL2737 (known locally as *Brasilera)* and cultivar CM4574. These varieties were selected
268 because they are suitable for growing in the region and because of their growth response to AMF
269 inoculation[8,25]. There were fourteen inoculation treatments with *R. irregularis.* This comprised the
270 parental isolates C2 and C3 and 3 and 9 single-spore progeny of the two parental isolates, respectively.
271 The treatments are summarized in Supplementary information table S3. Water was applied as a control
272 in a non-inoculated treatment. Both cassava cultivars were inoculated with each of the 14 inoculation or
273 the non-inoculated treatments in a randomized block design with nine blocks and one plant of each
274 cultivar per treatment per block. Two rows of plants were planted around each treated plant to reduce
275 edge effects and isolate treatments. Cassava was planted as stem cuttings (stakes) with the same
276 methodology and planting density as in Experiment 3. There was no artificial irrigation and conventional
277 crop management, for the region, was applied depending on pests, diseases and weed incidence.
278 Fertilizer was applied 45 days after planting and again at 90 days after planting. Plants in the first
279 experiment received 233 Kg ha^{-1} urea, 125 Kg ha^{-1} di-ammonium phosphates (DAP), 100 Kg ha^{-1}
280 potassium chloride (KCl). Plants in the second experiment received 84 Kg ha^{-1} DAP, 54 Kg ha^{-1} KCl, 41
281 Kg ha^{-1} of Kieserite (a fertilizer comprising 3% soluble potassium, 24% magnesium and 19% sulphur)
282 and 22 Kg ha^{-1} of Vicor® (a granular fertilizer comprising 15% nitrogen, 5% calcium, 3% magnesium,
283 2% sulphur, 0.02% boron, 0.02% copper, 0.02% manganese, 0.005% molybdenum, 2.5% zinc).
284 Inoculation of cassava was carried out 20 days after planting. Plants were inoculated with 500 AMF
285 propagules per plant. Each formulation with each AMF line was diluted with water to apply 10 ml to each
286 plant close to first roots produced by the plant. Non-inoculated plants received the same volume of

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287 water. The fungal inoculum was upscaled in an *in vitro* culture system by Mycovitro S.L. (Granada,
288 Spain) and suspended in an inert gel carrier[8].

289 **Plant and Fungal Growth Measurements:** Shoot biomass was collected and weighed directly in the
290 field (340 days after planting). Plant material was dried at 70°C until constant weight (approximately 49
291 hours). Cassava roots were collected and weighed in the field (360 days after planting) to measured
292 production per plant. Root dry weight was calculated using the specific gravity method after calibration[26].
293 Total AMF colonization in roots was measured in fine roots with a thickness of <2 mm. Fungal structures
294 were visualized with Schaeffer black ink[27]. The percentage of root colonization was determined by the
295 grid line-intersect method[21].

296 **Statistical Analyses:** All data were analyzed using the JMP® statistical discovery software (Statistical
297 Analysis Systems Institute, version 10). An analysis of variance was performed to analyze data from
298 both experiments. A mixed model was applied to test for significant differences between treatment on
299 cassava growth or AMF colonization. In that model *Cassava variety* and *AMF line* were fixed factors
300 and *Year* and *Block* were random factors. Restricted Maximum Likelihood (REML) was used to analyze
301 the model with random factors. Comparison of LS Means differences was accomplished using Tukey
302 honest significant difference or Student´s t test. To determine the variability explained by *Year* as a
303 factor, the same model was run without this factor. AMF colonization data was *arcsin* transformed before
304 statistical analysis

305 **Experiment 5: Experiment to test the amplitude of cassava root growth responses to multiple**
306 **single spore progeny of *R. irregularis* parental isolates in field conditions in Kenya and Tanzania**

307 **Experimental design:** The study was carried out in three locations: Ukwala-Kawayo, Siaya County,
308 Kenya (34° 10' 32.7" E; 00° 15' 12.1'' N); Kayenze, Biharamulo district, Tanzania (32°35'42.79"E; 02°
309 35' 20.41" S) and Kijuka, Sengerema district, Tanzania (31° 26' 37.18" E; 03° 12' 3.33" S). A total of 4
310 trials were conducted. One in Ukwala-Kawayo, two in Kayenze, and one in Kijuka. In the trial in Ukwala-
311 Kawayo, we used one local cassava land-race, known as Fumba Chai. In the two trials in Kayenze, we
312 used two varieties of cassava; a local land-race known as Mzao, and an improved cultivar known as
313 Mkombozi. In the trial in Kijuka, we used two varieties; a local land-race known as Mwanaminzi, and the
314 improved cultivar Mkombozi. The land-races were chosen because they are grown locally by farmers.

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315 The improved cultivars were recommended by IITA as they have been bred for resistance to diseases.
316 In Ukwala-Kawayo, we inoculated cassava with 14 different treatments with *R. irregularis*. These were:
317 Parental isolate C2 and 5 single spore progeny. We also used isolate C5 which has been shown to be
318 a clone of C2; Parental isolate C3 and 6 single spore progeny. In Kayenze and Kijuka, all plants were
319 inoculated with parental *R. irregularis* isolates A1, C2, B12, A5, C3. The isolate C5 (a clone of C2) was
320 also used. In addition, we inoculated cassava with up to 8 single-spore progeny of each of the parental
321 isolates. All parental isolates and single spore progeny of each parental line that were used at each
322 location and in each trial are shown in Supplementary information table S3). In addition to the plants
323 inoculated with *R. irregularis*, there were two control treatments; no inoculation and inoculation with the
324 carrier but with no fungus. Planting density was the same as in experiments 3 and 4. Each trial was
325 planted as a randomized block design with treated plants surrounded by 8 non-inoculated plants as in
326 experiment 4. There were 10 blocks in the trial in Ukwala-Kawayo and 12 blocks in all other trials. In
327 each trial, there was one plant of each treatment combination in each block so that block also
328 represented replicates. Methodology for planting and inoculation was the same as in experiment 4.
329 Fertilizers were applied between 25-45 days after planting (DAP). Plants received 150 $Kg.ha^{-1}$ N, 40
330 $Kg.ha^{-1}$ P and 180 $Kg.ha^{-1}$ K, according to the nutritional requirements proposed by the International
331 Institute of Tropical Agriculture (IITA). Plants were inoculated at planting, as in experiment 3, with 1g of
332 carrier diatomite containing 1000 fungal spores.

333 **Plant and fungal growth measurements:** At the final harvest we measured root fresh weight per plant
334 ($kg.plant^{-1}$) and AMF colonization in roots (% colonized root length). AMF colonization was estimated
335 using the grid-line intersection method[21]. Additionally, in order to have a standardized measure of
336 mycorrhizal effects on plants inoculated with different isolates, mycorrhizal responsiveness was
337 calculated[28].

338 **Statistical analyses:** Data was analysed using the R statistical software (R Core Team, 2018; version
339 3.5.1) and JMP® 13.2.0 (SAS institute Inc.). To test for significant differences among treatments
340 analysis of variance (ANOVA) was performed, using a post-hoc Tukey honest significant difference
341 (HSD) test. Where the data could not be assumed to be normally distributed we used the Wilcoxon
342 signed-rank test.

343 **Data availability**

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344 The data supporting the findings of this study are available from the corresponding author upon
345 reasonable request.

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411

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418 **Author affiliations**

419 *Department of Biology, National University of Colombia, Bogota, Colombia.*
420 Isabel Ceballos, Diego Camilo Peña-Quemba, Yuli M. Ordoñez, Alia Rodriguez

421 *Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland.*
422 Ivan D. Mateus, Ricardo Peña, Chanz Robbins, Pawel Rosikiewicz, Edward C. Rojas, Ian R. Sanders

423 *International Institute for Tropical Agriculture (IITA) Kenya, Nairobi, Kenya.*
424 Moses Thuita, Bernard Vanlauwe,

425 *Tanzania Agricultural Research Institute, Mwanza, Tanzania*
426 Peter Mlay Deusdedit,

427 *International Institute for Tropical Agriculture (IITA) Cameroon, Yaoundé, Cameroon.*
428 Cargele Masso

429 **Author contributions**

430 I.C. conceived and conducted experiment 4 and analysed data; I.D.M. conceived and conducted
431 experiments 1 and 2 and analysed the data; R.P. conceived and conducted experiment 5 and analysed
432 the data; D.C.P-Q. conceived and conducted experiment 3 and analysed the data; C.R. conducted

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433 experiment 3, Y.M.O. conducted experiment 4; E.C.J. conducted molecular analyses on fungal cultures
434 used as inocula; M.T. R.P. conceived and conducted experiment 5 and analysed the data; P.M.D.
435 conducugted part of experiment 5; C.M. conceived experiment 5, B.V. conceived experiment 5, A.R.
436 conceived experiments 3, 4 and 5, interpreted data and wrote the manuscript, IRS conceived all
437 experiments, interpreted at the data and wrote the manuscript.

438 **Competing interests**

439 The authors declare no competing financial interests.

440 **Materials and correspondence**

441 Correspondence or requests for materials to: ian.sanders@unil.ch

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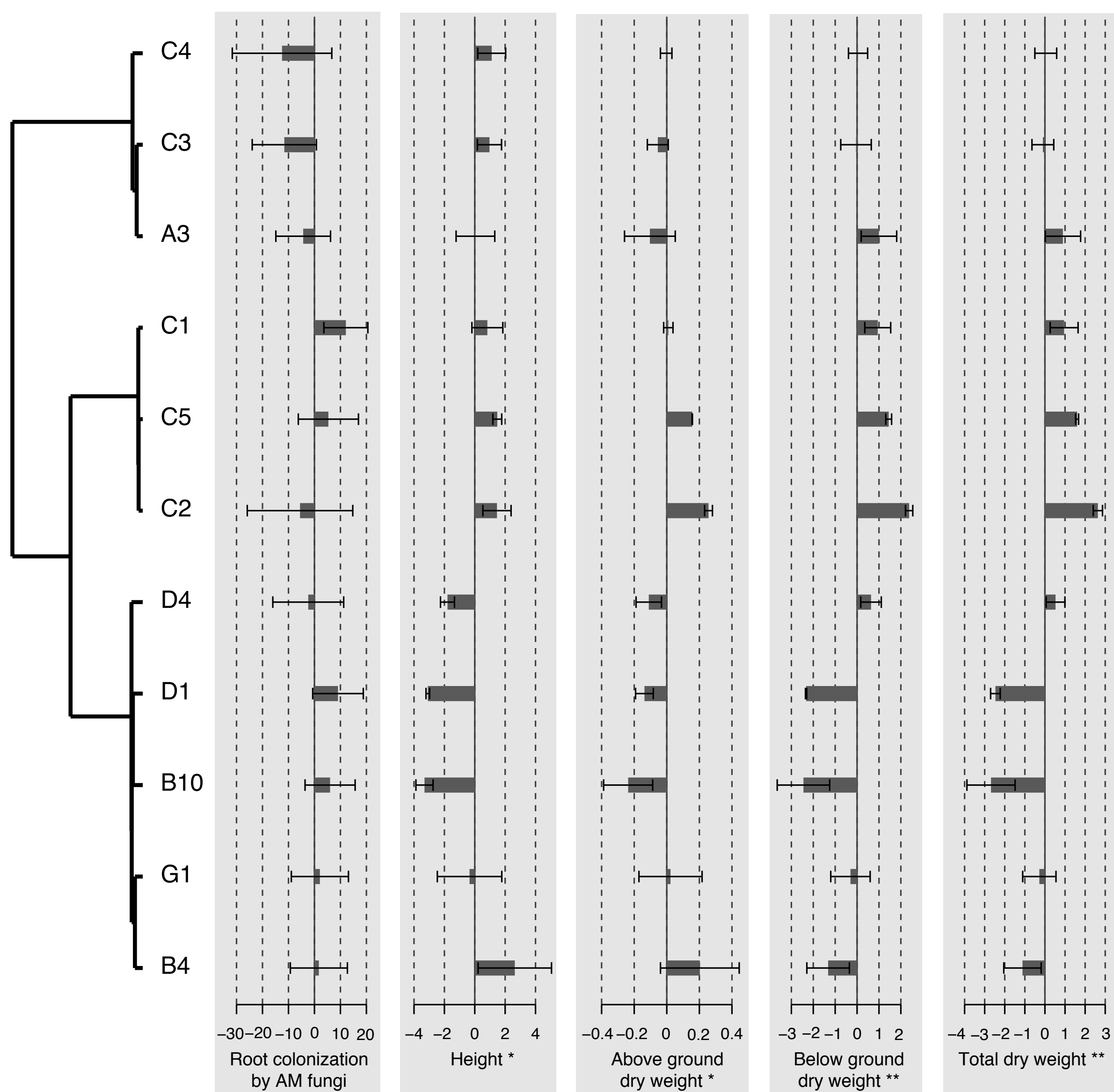
443 **Figure 1.** Variation in root colonization by AMF cassava height, shoot dry weight, root dry weight and
444 total cassava dry weight among plants inoculated with 11 genetically different isolates of *R. irregularis*
445 (experiment 2). Values shown are centred on the mean response across all treatments and bars
446 represent the standard deviation. Value of traits for fungal inoculation treatments are arranged according
447 to a dendrogram of genetic relatedness among fungal isolates. * $P \leq 0.05$; ** $P \leq 0.01$ represent
448 significance of tests of a phylogenetic signal of a given trait with at least one test. Statistical tests for
449 differences in a trait among treatments and tests for the existence of a significant phylogenetic signal
450 are given in Supplementary information table S1.



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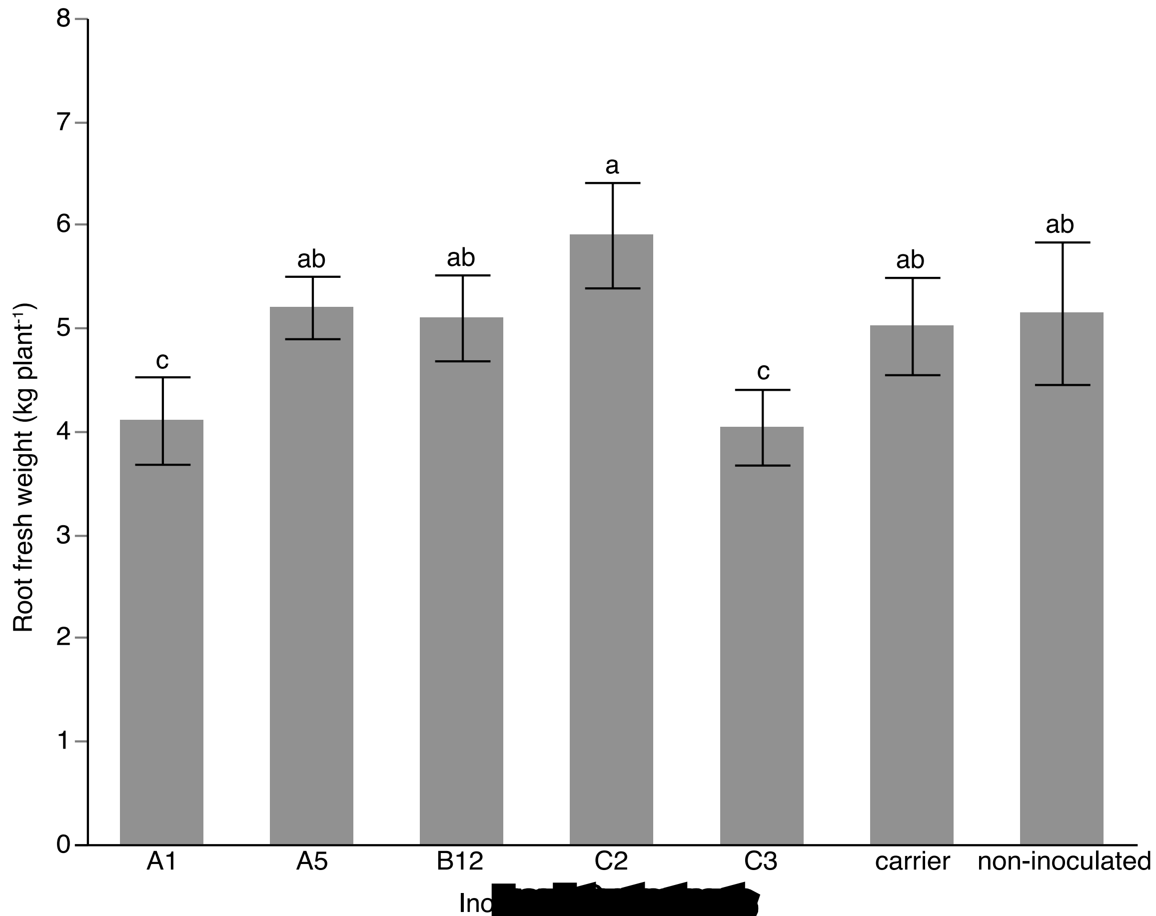
453 **Figure 2.** Mean root fresh weight per plant of cassava grown in normal farming conditions in Tauramena,
454 Colombia and with 7 different inoculation treatments (experiment 3). Each value represents the mean
455 of 6 replicate plots where the value o for each plot is the pooled mean of several plants. Bars represent
456 $\pm$1 S.E. Plants were inoculated with *R. irregularis* isolates A1, A5, B12, C2, C3 or were inoculated with
457 the carrier without *R. irregularis* or were not inoculated. Different letters represent significant differences
458 at $P \leq 0.1$. See Supplementary information table S2 for statistical analyses.



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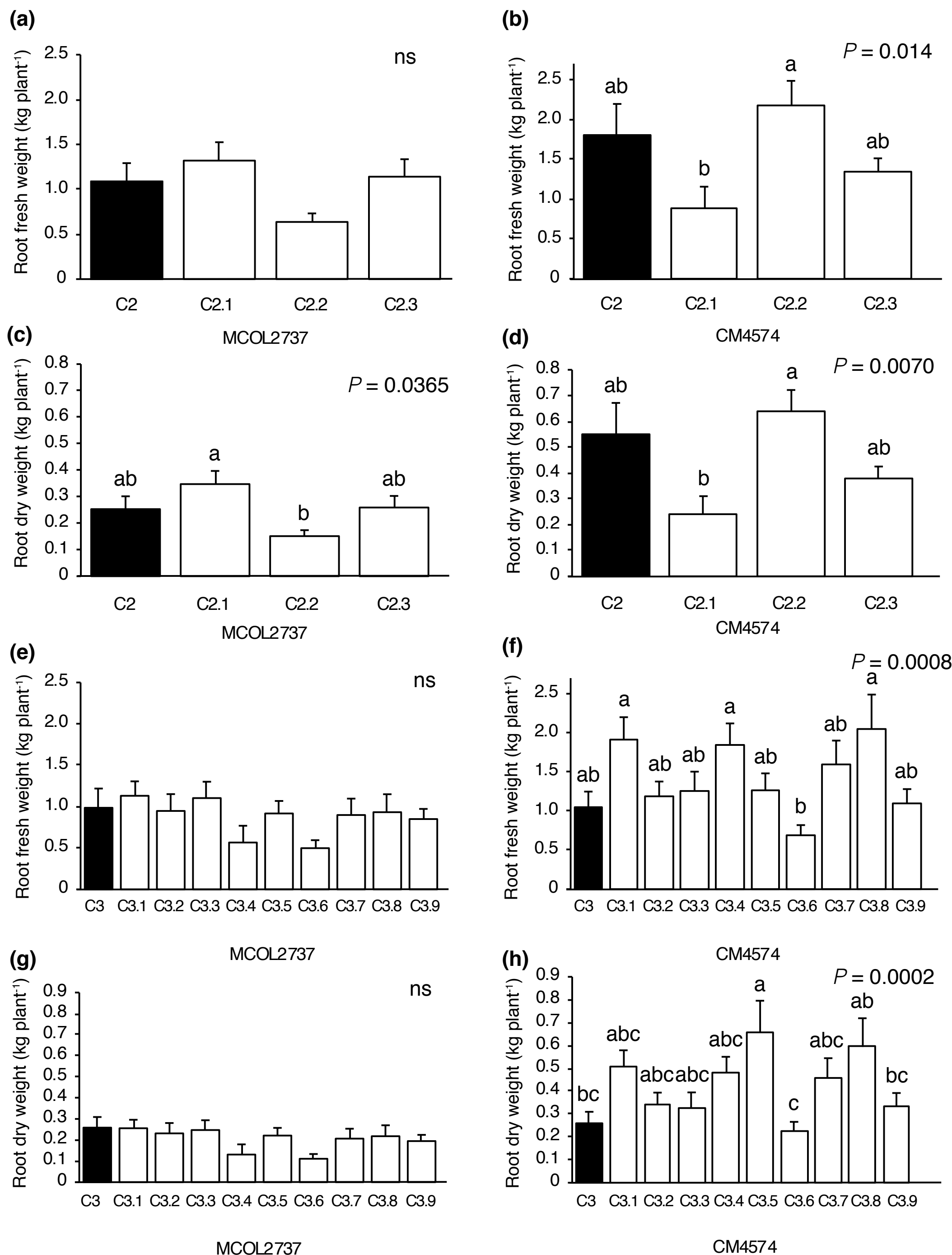
462 **Figure 3.** Mean root fresh and dry weight of cassava cultivars MCOL2737 and CM4574 inoculated with
463 the parental *R. irregularis* isolate C2 and its clonal progeny and the parental *R. irregularis* isolate C3
464 and its clonal progeny averaged over the two trials in experiment 4. Parental isolate treatments shown
465 in black and progeny treatments unshaded. Bars represent +1 S.E. Different letters above bars
466 represent significant differences ($P \leq 0.05$) according to a Tukey honest significant difference test. See
467 Supplementary information table S4 for a summary of statistical analyses and Supplementary
468 information for full details of all statistical tests.



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470 **Figure 4. (a)** Range of means of root fresh weight per plant (kg $plant^{-1}$) among cassava inoculated with a given parental *R. irregularis* isolate (A1, C2, B12, A5
471 & C3) and its respective progeny, in four trials conducted at three locations in Kenya and Tanzania. **(b)** Fold increase of cassava root weight in the most
472 productive treatment compared to the least productive in the same experiment. *Effect on root weight among plants inoculated with a parental AMF line and its
473 progeny was significant at $P \leq 0.05$. ND = no data. (c) shows the example of the treatments resulting in the largest difference in cassava root weight between
474 two sibling progeny of the same *R. irregularis* parent underlined in red in (a) and (b). (d) shows the example of the treatments resulting in the largest fold
475 difference in cassava root weight between two sibling progeny of the same *R. irregularis* parent underlined in purple in (a) and (b). Means for each comparison
476 between a parental R. irregularis isolate and its progeny with a given cassava cultivar and location is provided in Supplementary figures 4-7.
477

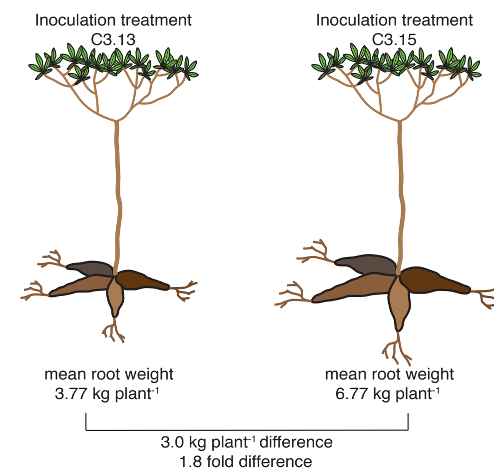
(a)

	Local variety					Improved variety				
	A1	**C2**	**B12**	**A5**	**C3**	**A1**	**C2**	**B12**	**A5**	**C3**
Ukwala-Kawayo	ND	1.05*	ND	ND	0.99*	ND	ND	ND	ND	ND
Kayenze trial 1	0.71	1.80*	0.91	1.66*	1.54*	0.50	1.02*	0.69	0.77*	0.40
Kayenze trial 2	1.59*	1.60*	1.51*	0.86	1.46*	0.94*	0.79	1.41*	1.02*	0.97*
Kijuka	1.97	0.95	2.25*	2.06*	3.00*	0.51	0.53	0.47	1.15*	0.59

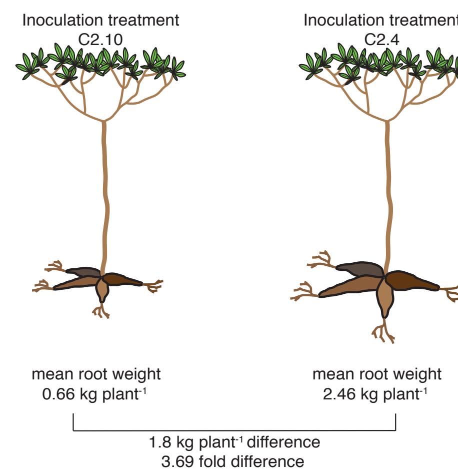
(b)

	Local variety					Improved variety				
	A1	**C2**	**B12**	**A5**	**C3**	**A1**	**C2**	**B12**	**A5**	**C3**
Ukwala-Kawayo	ND	1.87*	ND	ND	1.78*	ND	ND	ND	ND	ND
Kayenze trial 1	1.66	3.69*	1.81	3.65*	3.00*	1.79	2.94*	2.10	3.05*	1.65
Kayenze trial 2	2.17*	2.19*	1.89*	1.64	1.92*	2.45*	1.93	3.27*	2.26*	2.12*
Kijuka	1.46	1.19	1.47*	1.54*	1.80*	1.49	1.41	1.37	2.21*	1.47

(c) Treatments with largest and smallest mean root weights among C3 and its offsping treatments in Kijuka



(d) Treatments with largest and smallest mean fold change among C2 and its offsping treatments in Kayenze trial 1



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