Guanidine thiocyanate solution facilitates sample collection for plant rhizosphere microbiome analysis

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The interactions between rhizosphere microorganisms and plants are important for the health and development of crops. Analysis of plant rhizosphere bacterial compositions, particularly of those with resistance to biotic/abiotic stresses, mayimprove their applications in sustainable agriculture. Large-scale rhizosphere samplings in the field are usually required; however, such samples, cannot be immediately frozen. We found that the storage of samples at room temperature for 2 days leads to a considerable reduction in the operational taxonomic unit (OTU) number and the indices of bacterial alpha-diversity of rhizosphere communities. In this study, in order to overcome these problems, we established a method using guanidine thiocyanate solution for the preservation of rhizosphere samples after their collection. This method allowed the maintenance of the samples for at least 1 day at room temperature prior to their cryopreservation and was shown to be compatible with conventional DNA isolation protocols. Illumina sequencing of V3 and V4 hypervariable regions of the 16S rRNA gene was used to assess the feasibility and reliability of this method, and no significant differences were observed in the number of OTUs and in the Chao and Shannon indices between samples stored at -70°C and those stored in guanidine thiocyanate solution. Moreover, the representation of *Pseudomonas* spp. in samples stored in guanidine thiocyanate solution was not significantly different from that in samples stored at -70°C, as determined by real-time quantitative polymerase chain reaction ($p > 0.05$). Both types of samples were shown to cluster together according to principal coordinate analysis. Furthermore, guanidine thiocyanate solution did not affect the bacterial taxon profiles at different storage periods compared with those observed when storing the samples below -70°C. Even incubation of thawed samples (frozen at -70°C) for 15 min at room temperature induced minor changes in the bacterial composition. Taken together, our results demonstrated that guanidine thiocyanate solution may provide a reliable alternative for the preservation of rhizosphere samples in the field.

1   **Guanidine Thiocyanate Solution Facilitates Sample Collection for Plant Rhizosphere**

2   **Microbiome Analysis**

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12 **ABSTRACT**

13 The interactions between rhizosphere microorganisms and plants are important for the health and

14 development of crops. Analysis of plant rhizosphere bacterial compositions, particularly of those

15 with resistance to biotic/abiotic stresses, may improve their applications in sustainable agriculture.

16 Large-scale rhizosphere samplings in the field are usually required; however, such samples, cannot

17 be immediately frozen. We found that the storage of samples at room temperature for 2 days leads

18 to a considerable reduction in the operational taxonomic unit (OTU) number and the indices of

19 bacterial alpha-diversity of rhizosphere communities. In this study, in order to overcome these

20 problems, we established a method using guanidine thiocyanate solution for the preservation of

21 rhizosphere samples after their collection. This method allowed the maintenance of the samples

22 for at least 1 day at room temperature prior to their cryopreservation and was shown to be

23 compatible with conventional DNA isolation protocols. Illumina sequencing of V3 and V4

24 hypervariable regions of the 16S rRNA gene was used to assess the feasibility and reliability of

25 this method, and no significant differences were observed in the number of OTUs and in the Chao

26 and Shannon indices between samples stored at -70°C and those stored in guanidine thiocyanate

27 solution. Moreover, the representation of *Pseudomonas* spp. in samples stored in guanidine

28 thiocyanate solution was not significantly different from that in samples stored at -70°C, as

29 determined by real-time quantitative polymerase chain reaction ($p > 0.05$). Both types of samples

30 were shown to cluster together according to principal coordinate analysis. Furthermore, guanidine

31 thiocyanate solution did not affect the bacterial taxon profiles at different storage periods compared

32 with those observed when storing the samples below -70°C. Even incubation of thawed samples

33 (frozen at -70°C) for 15 min at room temperature induced minor changes in the bacterial

34 composition. Taken together, our results demonstrated that guanidine thiocyanate solution may

35 provide a reliable alternative for the preservation of rhizosphere samples in the field.

36 **INTRODUCTION**

37 Plant-associated bacteria, particularly rhizosphere bacteria, play important roles in plant growth.

38   Some rhizosphere bacteria produce different compounds, such as nutrients and hormones, that

39   promote the growth of plants; moreover, some are soil-borne pathogens that can lead to a dramatic

40   yield loss, whereas others are neutral (*Blom et al., 2011*). Plant rhizospheres have been shown to

41   contain approximately 10^{11} bacterial cells per gram of root, more cells than in the phyllosphere

42   and soil (*Egamberdieva et al., 2008*). Plant-growth-promoting rhizobacteria (PGPR) are a large

43   bacterial group, first identified as the bacteria enhancing plant productivity and immunity

44   (*Lugtenberg and Kamilova, 2009*). However, several recent studies have demonstrated that PGPR

45   induce physical and chemical changes in plants, increasing their tolerance to salinity (*Tank and*

46   *Saraf, 2010*), drought (*Vurukonda et al., 2016*), cold (*Kakar et al., 2016*), and heavy metals (*Ullah*

47   *et al., 2015*). Furthermore, PGPR may increase nitrate and phosphate uptake from soil (*Pii et al.,*

48   *2015*), thereby helping reduce the use of fertilizer and water contamination. Most importantly, the

49   investigation of the interactions between rhizosphere bacteria and plants will help promote their

50   use in agriculture.

51   Currently, both culture-dependent and -independent methods are used for analysis of

52   rhizosphere bacteria. In the past, denaturing gradient gel electrophoresis (DEEG) has been widely

53   used as a culture-independent method for analysis of microbial community structure; however,

54   high-throughput sequencing has been developed as a more sensitive technique and is now

55   commonly used. Thousands of operational taxonomic units (OTUs) are obtained by high-

56   throughput sequencing (*Lundberg et al., 2012*; *Bulgarelli et al., 2013*; *Turner et al., 2013*), whereas

57   DGGE usually only yields dozens of OTUs *(Vallaeys et al., 1997).*

58   From the collection of samples to the sequencing of 16S rRNA genes, many factors, such as

59   template preparation (*Tian et al., 2017*), storage conditions (*Flores et al., 2015*), amplification

60   primers (*Klindworth et al., 2013*), and sequencing technologies (*Tremblay et al., 2015*), affect the

61   alpha and beta diversity of bacterial communities of samples, and cause inaccurate analysis of rare

62   taxa. However, the initial issue affecting microbiome analysis is variations in bacterial

63   composition due to outdoor temperature. Previous studies have reported that delayed freezing has

64   a considerable effect on bacterial communities in soil samples (*Rublin et al., 2013*) and

65   human stool samples (*Flores et al., 2015*). Some reports have indicated that short-term incubation

66   at room temperature has no obvious effects on the composition and diversity of microbial

67   communities, particularly for human fecal samples (*Tedjo et al., 2015; Guo et al., 2016)*. Several

68   groups have reported that incubation at room temperature does not affect the overall bacterial

69   community structure of soil samples (*Dolfing et al., 2004*; *Lauber et al., 2010*). However, to the

70   best of our knowledge, few studies have evaluated these features in plant rhizosphere samples,

71   which are more diverse than soil samples (*Lundberg et al., 2012*). Accordingly, it is reasonable to

72   hypothesize that outdoor temperature may have different effects on plant rhizosphere samples.

73   Immediate freezing of samples is usually not possible in the field, particularly in remote areas.

74   Additionally, the use of liquid nitrogen or ice in a portable insulated container is also not practical

75   when large-scale samples are collected. Thus, novel methods are needed to overcome these

76   problems.

77   Two recent reports indicated guanidine thiocyanate (GTC) solution promotes a stability in

78   composition and diversity of human fecal microbial analysis at room temperature form one day to

79   one week (*Nishimoto et al., 2016*; *Hosomi et al., 2017*). Guanidine isothiocyanate denatures RNase

80   and DNase, inhibits the growth of bacteria (*Chomczynski and Sacchi, 2006*; *Hisada et al., 2015*),

81   and is widely used in DNA isolation (*Stearns et al., 2015*; *Neubeck et al., 2017*) and the

82   preservation of human stool samples. However, this compound has not been applied for analysis

83   of the plant rhizosphere microbiome to date.

84   In this study, we examined the effects of GTC solution on prevention of alterations in bacterial

85   taxa and assessed the feasibility of this approach for analysis of the rhizosphere microbiome. The

86   objective of this study was to determine whether GTC buffer could be used to preserve soil samples

87   at room temperature for at least 1 day prior to additional cryopreservation. We expect that this

88   method may have applications in sampling large-scale rhizosphere soil in fields without requiring

89   immediate freezing. Here, we employed the Illumina MiSeq of 16S rRNA amplicon to assess the

90   feasibility of using GTC solution, and attempted to develop a simple and reliable method for

91   storing plant rhizosphere samples in field.

92   **MATERIALS AND METHODS**

93   **Study system**

94   Peanut (*Arachis hypogaea* L.), the world's fourth largest oilseed crop, is an allotetraploid (AABB,

95   2n = 40) originating from *A. duranensis* (A genome) and *A. ipaensis* (B genome) (*Seijo et al.,*

96   *2007*). Deciphering the rhizosphere microbiome of *A. hypogaea* L. can not only help us fully

97   understand the plant-microbe interactions to improve plant health and yield, but may also provide

98   insights into the control of subterranean insect pests (*Geng et al., 2018*).

99   **Rhizosphere compartment sampling**

100   The peanut cultivar *Huayu22* was provided by Shandong Peanut Research Institute and cultivated

101   in a greenhouse with a nutritional soil and vermiculite mixture (volume ratio 2:1, pH = 5.23,

102   organic matter content = 222 g kg^{-1}, total nitrogen = 7.00 g kg^{-1}) as in our previous study (*Geng*

103   *et al., 2018*), at 28°C with 60% relative humidity and a 14-h photoperiod. Rhizosphere soil was

104   sampled as previously described by *Bulgarelli et al.* (2013) with minor changes. Loose soil was

105   removed from the roots by shaking. Peanut roots were cut off using sterile scissors, transferred to

106   sterile 50-mL plastic centrifuge tubes containing 40 mL phosphate-buffered saline (PBS; pH 7.0,

107   8 μL Silwet L-77), and soaked for 5 min. The samples were then vortexed for 15 s. Subsequently,

108   roots were removed, and the rhizosphere soil suspension was passed through a sieve with 100

109   openings per square inch and centrifuged at 3,200 ×*g* for 15 min. Individual rhizosphere soil

110   samples were collected from nine peanut plants. Three rhizosphere soil samples from three plants

111   were homogenized as one sample. Each sample was then divided into nine parts (100 mg each)

112   and stored under different conditions (Fig. 1): 1) at room temperature (25°C, RT sample); 2) frozen

113   at -70°C (FR sample); and 3) supplemented with 100 μL GTC buffer, containing 100 mM Tris-

114   HCl (pH 9), 40 mM ethylenediaminetetraacetic acid, 4 M GTC, and 0.001% bromothymol, stored

115   at room temperature for 24 h, and then frozen at -20°C (GTC sample). For the GTC and RT

116   treatments, bacterial genomic DNA was extracted at different time points (days 2, 8, and 31

117   following the collection). For the FR samples, bacterial genomic DNA was extracted on day 2

118   after initiation of the experiment, and the thawed samples were kept at room temperature for 15

119   min before isolation on days 8 and 31.

Extraction of bacterial genomic DNA

121   Genomic DNA was extracted from rhizosphere soils using a PowerSoil DNA Isolation Kit (MoBio

122   Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. For the GTC

123   treatment, the suspension was centrifuged at 5000 $\times g$ for 15 min, and the GTC buffer was removed.

124   Soil samples were washed once with 1 mL PBS and centrifuged at 5000 $\times g$ for 15 min to remove

125   PBS.

GTC buffer and DNA extraction

127   The effects of GTC buffer on DNA extraction were analyzed. Rhizosphere soil samples were

128   collected as described above and divided into 15 parts (100 mg each). The soil samples were

129   treated as follows, with three repetitions per treatment: one of the treatments was stored at -70°C

130   without GTC buffer (N-GTC), and the other four treatments were stored at -20°C after adding 100

131   μL GTC buffer. Before DNA extraction, GTC buffer was removed by centrifugation at 3,200 $\times g$

132   for 15 min and washed without PBS (GTC-W0) or washed once (GTC-W1), twice (GTC-W2), or

133   three times (GTC-W3) with 1 mL PBS. Genomic DNA was extracted from the soil samples using

134   a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the

135   manufacturer's instructions. The DNA concentration was determined using a NanoDrop

136   2000/2000c (Thermo Fisher Scientific, Rockford IL, USA). Copy numbers of the 16S rRNA genes

137   in these samples were determined by real-time quantitative polymerase chain reaction (qPCR).

138   Data were analyzed using one-way ANOVA (Tukey's HSD) with SPSS version 19.0 (SPSS, Inc.,

139   Chicago, IL, USA).

16S rRNA sequencing and downstream data analysis

141   Next-generation sequencing library preparations and Illumina MiSeq sequencing were conducted

142   at Biomarker, Inc. (Beijing, China). A part of the hypervariable region of 16S rRNA (V3+V4

143   region) was amplified using universal primers with barcodes (Table S1, 341F: 5′-

144   CCTAYGGGRBGCASCAG-3′, and 806R: 5′-GGACTACNNGGGTATCTAAT-3′). PCR was

145   performed using Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA,

146   USA), and PCR products were purified using a Qiagen Gel Extraction Kit (Qiagen, Germany).

147   Sequencing libraries were prepared using a TruSeq DNA PCR-Free Sample Preparation Kit

148   (Illumina, San Diego, CA, USA) following the manufacturer's protocol. The library quality was

149   evaluated on a Qubit @ 2.0 Fluorometer (Thermo Fisher Scientific) and Agilent Bioanalyzer 2100

150   system (Agilent Technologies, Palo Alto, CA, USA). The library was sequenced on an Illumina

151   HiSeq 2500 platform (Illumina), yielding 250-bp paired-end reads.

152   Quality-filtering using Trimmomatic (version 0.36) (*Bolger et al., 2014*) was performed with the

153   following criteria: (i) bases off the start and end of a read below a threshold quality (score < 3)

154   were removed; (ii) and the reads were truncated at any site receiving an average quality score of

155   less than 5 over a 4-bp sliding window, discarding the truncated reads that were shorter than 100

156   bp. Clean reads were compared with Gold database (Broad Microbiome Utilities, version

157   microbiomeutil-r20110519) using UCHIME, and chimeric reads were filtered to obtain the

158   effective reads (*Edgar et al., 2011*). Sequences with ≥97% similarity were clustered into the same

159   OTUs using USEARCH software (version 9.2.64) (*Edgar, 2013*). Representative OTU sequences

160   were selected, and a 16S rRNA OTU table and abundance data were generated (Table S2).

161   Additionally, these representative sequences were annotated to species according to the Ribosomal

162   Database Project classifier (version 2.2) and the Greengenes (version 13.8) (*DeSantis et al., 2006*).

163   Alpha-diversity indices were calculated using USEARCH software and data were analyzed using

164   one-way ANOVA (LSD) with SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA) to compare

165   various groups with each other. Weighted UniFrac distances were generated using relative

166   abundance (*Lozupone and Knight, 2005*). Principal coordinate analysis (PCoA) and ANOSIM

167   based on weighted UniFrac distances were performed using R software (version 3.4.0). Pearson

168   correlation coefficients were additionally calculated using the OTU abundance data and SPSS

169   Statistics 24 (IBM, USA) (*Nishimoto et al., 2016*). Fold changes in the taxonomic abundances of

170   the top 15 genera between GTC/RT and FR-2d treatments were calculated, and the data were

171   analyzed using Wilcoxon signed-rank test (SPSS Statistics 24, IBM, USA).

172   **Quantitative real-time PCR**

173   Real-time qPCR analysis was performed using a 7500 Real-Time PCR System (Applied

174   Biosystems, Foster City, CA, USA) with SYBR Premix Dimer Eraser (Perfect Real Time; Takara

175   Bio, Otsu, Japan). The reaction conditions were as follows: 94°C for 15 min, followed by 40 cycles

176   of 94°C for 10 s and 61°C for 32 s. Reactions were performed in triplicate. A ~140-bp fragment

177   of the 16S rRNA gene was enzymatically amplified with primers 357F (5'-

178   CTCCTACGGGAGGCAGCAG -3') and 519R (5'- GWATTACCGCGGCKGCTG -3') (*Lane,*

179   *1991*). Another fragment of the 16S rRNA gene of *Pseudomonas* spp. was amplified with a specific

180   primer pair: Ps280F (5'- TAACTGGTCTGAGAGGATGATCAGT -3') and Ps441R (5'-

181   CCCAACTTAAAGTGCTTTACAATCC -3') (*Geng et al., 2018*). The relative abundance was

182   calculated by dividing the number of copies of the 16S rRNA gene of *Pseudomonas* spp. by the

183   total number of 16S rRNA gene copies for all species. Data were analyzed using one-

184   way ANOVA (Tukey's HSD) with SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA).

185   **RESULTS**

186   **Effects of GTC buffer on DNA concentration**

187   To explore the effects of GTC buffer on the DNA concentrations of rhizosphere samples, we

188   designed five different treatments for implementation before total DNA isolation. Compared with

189   the control, GTC buffer caused a 75.3% decrease in the DNA concentration ($p < 0.05$, Fig. 2A);

190   washing two or three times with PBS also significantly reduced the DNA concentration ($p < 0.05$,

191   Fig. 2A), whereas washing once with PBS did not affect the DNA concentration ($p > 0.05$, Fig.

192   2A). Copy number of the 16S rRNA gene transcript were determined by real-time qPCR. The

193   results showed that washing once with PBS did not affect copy number of the 16S rRNA gene

194   transcript, whereas the use of GTC buffer or washing two or three times with PBS led to a

195   significant decrease in the copy number of the 16S rRNA gene transcript (Fig. 2B). Based on the

196   above results, we washed the samples preserved with GTC with PBS once before extracting total

197   DNA using the PowerSoil DNA Isolation Kit.

198   **Room-temperature storage affected the diversity of rhizosphere bacterial communities**

199   Following quality control and assembly, 56,214 to 62,335 sequences of the V3 and V4 variable

200   regions of the 16S rRNA gene were obtained from each sample, and data were deposited in the

201   NCBI SRA database under accession no. SRP134256 (Table 1). The relative abundances of the

202   different taxa in all samples are presented in Table S2. The number of high-quality sequences for

203   samples stored at room temperature ranged from 58,342 to 62,335. No significant differences in

204   the number of high-quality sequences were observed between the RT-2d and FR-2d samples, but

205   the average number of OTUs obtained for the RT group was found to be significantly lower than

206   that obtained for the FR treatment (from $1,329 \pm 128$ to $2,154 \pm 79$; $p < 0.05$; Table 1). Compared

207   with that obtained for the FR group, considerable decreases in the Chao and Shannon indices was

208   observed in the RT group.

209   **GTC and FR samples yielded similar taxonomic and abundance profiles**

210   GTC was used at the final collection step and then removed by centrifugation before DNA

211   isolation. An increase in the number of high-quality sequences between GTC-31d and FR-2d

212   samples was observed. GTC-2d and -31d samples also showed a higher number of OTUs and the

213   Chao and Shannon indices than that of FR-2d samples (Table 1; $p < 0.05$). Proteobacteria

214   represented the dominant component of the peanut rhizosphere in all treatments (Table S2).

215   Furthermore, fold changes in the taxonomic abundances of the top 15 genera between GTC and

216   FR-2d treatments were calculated (Fig. 3C), and no significant differences were obtained in the

217   Wilcoxon signed-rank test ($p = 0.226$, median $= 0.086$). However, significant changes were

218   obtained at the genus level between RT and FR-2d groups (Fig. 3B; Wilcoxon signed-rank test, p

219   < 0.001, median $= -1.585$). The relative abundance of *Pseudomonas* spp. in all samples was

220   analyzed by real-time qPCR. The representation of *Pseudomonas* spp. in all GTC samples was not

221   significantly different from that in FR-2d samples ($p > 0.05$, Fig. 4) but was significantly lower

222   than that in all RT-2d and 31d samples ($p < 0.05$, Fig. 4). These results were consistent with the

223   illumina sequencing results.

224   PCoA was used to characterize differences in the rhizosphere bacterial community structure

225   between all analyzed groups (Fig. 5), showing a clear clustering of the samples. FR-2d and all

226   GTC samples were shown to cluster together, indicating similar bacterial community

227   compositions, which was consistent with the previously presented Pearson's correlation analysis.

228   The first principal coordinate (PC1) contributed to 60% of the total variation and revealed that the

229   structure of the bacterial community in the RT samples differed from those in the FR-2d and GTC

230   samples. The main factors in PC2 were found to be different time points in the RT samples, which

231   contributed to approximately 19% of the variation.

232   **DISCUSSION**

233   Many studies have shown that various plants (*Lundberg et al., 2012*; *Bulgarelli et al., 2013*; *Turner*

234   *et al., 2013*) influence distinct rhizosphere bacterial communities, particularly beneficial bacteria,

235   such as those that can promote biomass synthesis (*Rodriguez et al., 2008*), or increase resistance

236   to bacterial (*Rudrappa et al., 2008*; *Lakshmanan et al., 2012*) and fungal plant pathogens (*Mavrodi*

237   *et al., 2012a*; *b*). These studies have demonstrated that rhizosphere microorganisms may play

238   important roles in future efforts to develop sustainable agriculture. To this end, investigation of

239   the interactions between rhizosphere microbiome and plants is crucial.

240   In the present study, we developed a simple collection method using GTC solution to prevent

241   changes in the bacterial community composition in plant rhizosphere samples at room temperature;

242   this approach was shown to be an alternative method for the freezing of samples when sampling

243   in the field. Pyrosequencing of 16S rRNA amplicons was used to evaluate the feasibility of the

244   method, compare its effectiveness with that of freezing, and determine the reproducibility between

245   different time intervals. The structure and OTUs of all GTC samples were shown to cluster together

246   in the PCoA analysis (Fig. 5), indicating that, in contrast to previously reported results (*Flores et*

247   *al., 2015*), the removal of the GTC buffer before DNA isolation using a PowerSoil DNA Isolation

248   Kit did not affect the bacterial composition of the sample and that no differences in rhizosphere

249   bacterial community were found between samples treated with GTC and samples subjected to

250   freezing.

251   To further analyze the effects of storage condition on the overall taxonomic compositions of

252   rhizosphere samples and reproducibility of GTC preservation, Pearson's correlation coefficients

253   of the relative abundance of all OTUs in all treatment groups were analyzed (Fig. S1). Remarkably,

254   the relative OTU abundance in the GTC-31d sample showed high correlations ($r \geq 0.962$, $p < 0.01$)

255   with those in the GTC-2d and GTC-8d groups. However, the OTU abundance at different time

256 points in RT groups was shown to be less correlated ($r \leq 0.825$, $p < 0.01$). All abundances obtained

257 for the GTC groups were shown to correlate more ($r \geq 0.909$, $p < 0.01$) with those determined in

258 the FR-2d groups, than those of the RT groups ($r \leq 0.479$, $p < 0.01$). However, the correlation

259 between the FR samples incubated for 15 min at room temperature before DNA extraction and the

260 control samples ranged from 0.854 to 0.975 ($p < 0.01$).

261 This further illustrated that storing of the samples in GTC may represent an alternative to the

262 frozen preservation of rhizosphere soil samples. Furthermore, the storage period in GTC did not

263 affect the rhizosphere taxon profiles. This demonstrated that the reproducibility of the GTC-

264 associated analyses was good. GTC preservation may help elucidate the bacterial community

265 profiles of rhizosphere samples collected in the field.

266 According to our results, it is not recommended to store rhizosphere samples at room

267 temperature regardless of the length of time. We found that the OTU number and alpha-diversity

268 indices of the rhizosphere samples maintained at room temperature significantly decreased (Table

269 1, $p < 0.05$), most likely due to the degradation of bacterial DNA at room temperature. And the

270 composition of these samples was also affected by changes in the abundance of individual taxa.

271 Our findings are consistent with the majority of previous reports (*Rublin et al., 2013; Flores et al.,*

272 *2015*). Thus, the thawed FR-8d and 31d samples even incubated at room temperature for 15 min

273 prior to DNA isolation may lead to the alterations in the bacterial communities (Fig. 5, Fig. S1),

274 suggesting that even for frozen samples, DNA should be extracted immediately after thawing.

275 **CONCLUSIONS**

276 Taken together, our results established a method that allowed the samples to be stored at room

277 temperature for at least 1 day prior to cryopreservation. GTC solution was found to effectively

278 preserve rhizosphere soil samples at room temperature, eliminating the requirement of immediate

279 freezing of rhizosphere soil samples during large-scale sampling in fields. This method did not

280 affect the bacterial community structure and diversity of the rhizosphere soil. Accordingly, our

281 novel approach may facilitate sample collection for plant rhizosphere microbiome analysis on a

282 large scale and promote the investigation of plant-microbe interactions.

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403

404   **Figure legends**

405   **Figure 1** Flow chart showing experimental design. The samples were stored and processed under

406   different conditions: RT samples, at room temperature; FR samples, at -70°C; GTC samples, stored

407   with 100 μL guanidine thiocyanate for 24 h at room temperature and maintained at -20°C long-

408   term. All samples thawed for 10 min and after that, FR-8d (RT) and FR-31d (RT) were kept in

409   room temperature for another 15 min.   DNA was isolated at day 2, 8, and 31.

410   **Figure 2** DNA concentration (A) and number of copies of 16S rRNA gene (B) of peanut

411   rhizosphere samples from five different treatments. The five different treatments were performed

412   as follows before DNA extraction: rhizosphere samples were collected and preserved in GTC

413   buffer (named GTC-W0) and then washed with PBS buffer one (GTC-W1), two (GTC-W2), or

414   three (GTC-W3) times; rhizosphere samples without GTC buffer were used as positive controls

415   (N-GTC). n = 3. The error bars represent standard deviation. Different lower-case letters indicate

416   significant differences between groups analyzed by one-way ANOVA (Tukey's HSD) ($p < 0.05$).

417   **Figure 3** The distribution of top 15 genera in the FR, GTC, and RT groups (A). Comparisons of

418   the taxonomic abundances of top 15 genera between FR and RT groups (B), and FR and GTC

419   groups (C). FR-2d, rhizosphere samples stored at -70°C for 2 days (n = 3). GTC-2d, -8d, and 31d

420   are samples stored in 100 μL of GTC guanidine thiocyanate at room temperature for 24 h, and -

421   20° for 1, 7, and 30 days, respectively (n = 3). RT-2d, -8d, and 31d are samples stored at room

422   temperature for 2, 8, and 31 days, respectively (n = 3). The error bars represent standard deviation.

423   **Figure 4** Relative abundance of Pseudomonas spp. in all rhizosphere samples of peanuts, as

424   determined by real-time qPCR and pyrosequencing. FR-2d, rhizosphere samples stored at -70°C

425   for 2 days (n = 3). FR-8d (RT) and FR-31d (RT) are rhizosphere samples stored at -70°C for 8 and

426   31 days, respectively, thawed at room temperature for 15 min before DNA isolation (n = 3). GTC-

427   2d, -8d, and 31d are samples stored in 100 μL of GTC guanidine thiocyanate at room temperature

428   for 24 h, and -20° for 1, 7, and 30 days, respectively (n = 3). RT-2d, -8d, and 31d are samples

429   stored at room temperature for 2, 8, and 31 days, respectively (n = 3). The error bars represent

430   standard deviation. [a,b,c] Different lower-case letters indicate significant differences between groups

431   analyzed by one-way ANOVA (Tukey's HSD) analysis ($p < 0.05$).

432   **Figure 5** Weighted UniFrac principal coordinate analysis (PCoA) of the rhizosphere bacterial

433   community structure following the different treatments. FR-2d, rhizosphere samples stored at -

434   70°C for 2 days (n = 3). FR-8d (RT) and FR-31d (RT) are rhizosphere samples stored at -70°C for

435   8 and 31 days, respectively, thawed at room temperature for 15 min before DNA isolation (n = 3).

436   GTC-2d, -8d, and 31d are samples stored in 100 µL of GTC guanidine thiocyanate at room

437   temperature for 24 h, and -20° for 1, 7, and 30 days, respectively (n = 3). RT-2d, -8d, and 31d are

438   samples stored at room temperature for 2, 8, and 31 days, respectively (n = 3). Ellipses, 95%

439   confidence intervals.

440   **Figure S1** Pearson correlation coefficients showing the relative abundance of different operational

441   taxonomic units (OTUs) in the analyzed samples. FR-2d, rhizosphere samples stored at -70°C for

442   2 days (n = 3). FR-8d (RT) and FR-31d (RT) are rhizosphere samples stored at -70°C for 8 and 31

443   days, respectively, thawed at room temperature for 15 min before DNA isolation (n = 3). GTC-2d,

444   -8d, and 31d are samples stored in 100 µL of GTC guanidine thiocyanate at room temperature for

445   24 h, and -20° for 1, 7, and 30 days, respectively (n = 3). RT-2d, -8d, and 31d are samples stored

446   at room temperature for 2, 8, and 31 days, respectively (n = 3).

Figure 1

Flow chart showing experimental design.

The samples were stored and processed under different conditions: RT samples, at room temperature; FR samples, at -70°C; GTC samples, stored with 100 μL guanidine thiocyanate for 24 h at room temperature and maintained at -20°C long-term. All samples thawed for 10 min and after that, FR-8d (RT) and FR-31d (RT) were kept in room temperature for another 15 min. DNA was isolated at day 2, 8, and 31.

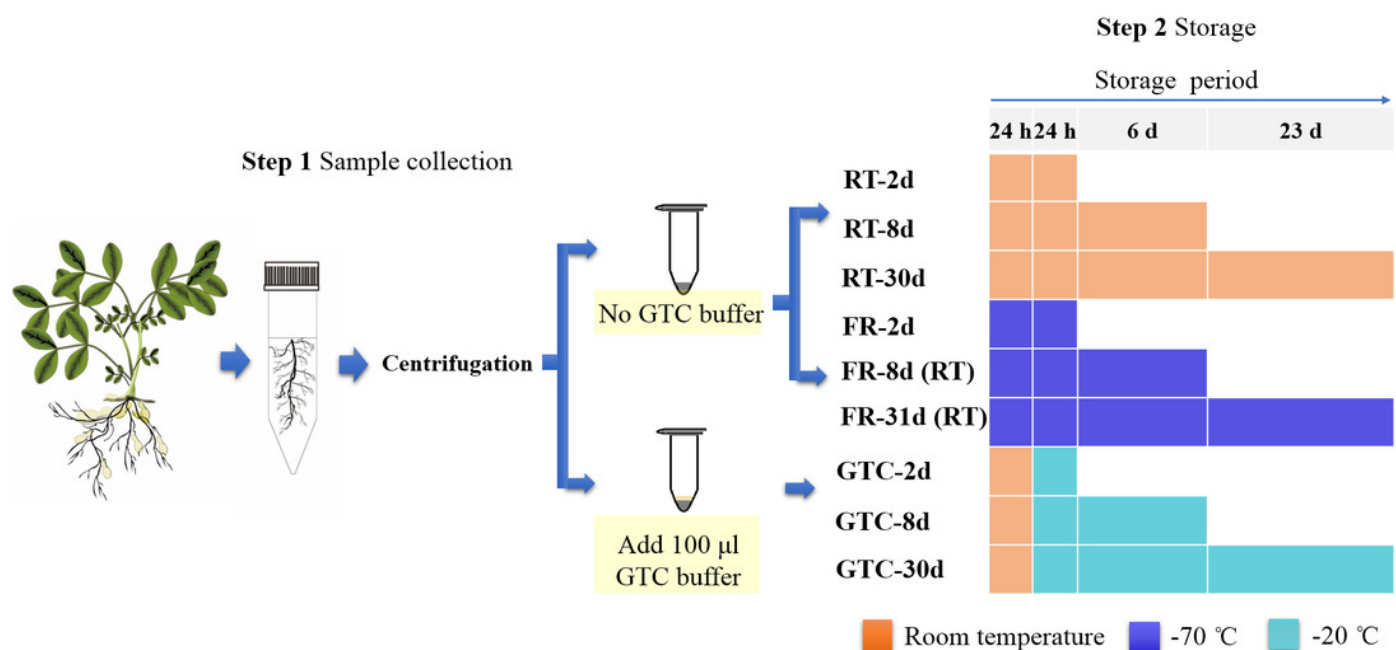



Figure 2

DNA concentration (A) and number of copies of 16S rRNA gene (B) of peanut rhizosphere samples from five different treatments.

The five different treatments were performed as follows before DNA extraction: rhizosphere samples were collected and preserved in GTC buffer (named GTC-W0) and then washed with PBS buffer one (GTC-W1), two (GTC-W2), or three (GTC-W3) times; rhizosphere samples without GTC buffer were used as positive controls (N-GTC). n = 3. The error bars represent standard deviation. Different lower-case letters indicate significant differences between groups analyzed by one-way ANOVA (Tukey's HSD) ($p < 0.05$).

A

B

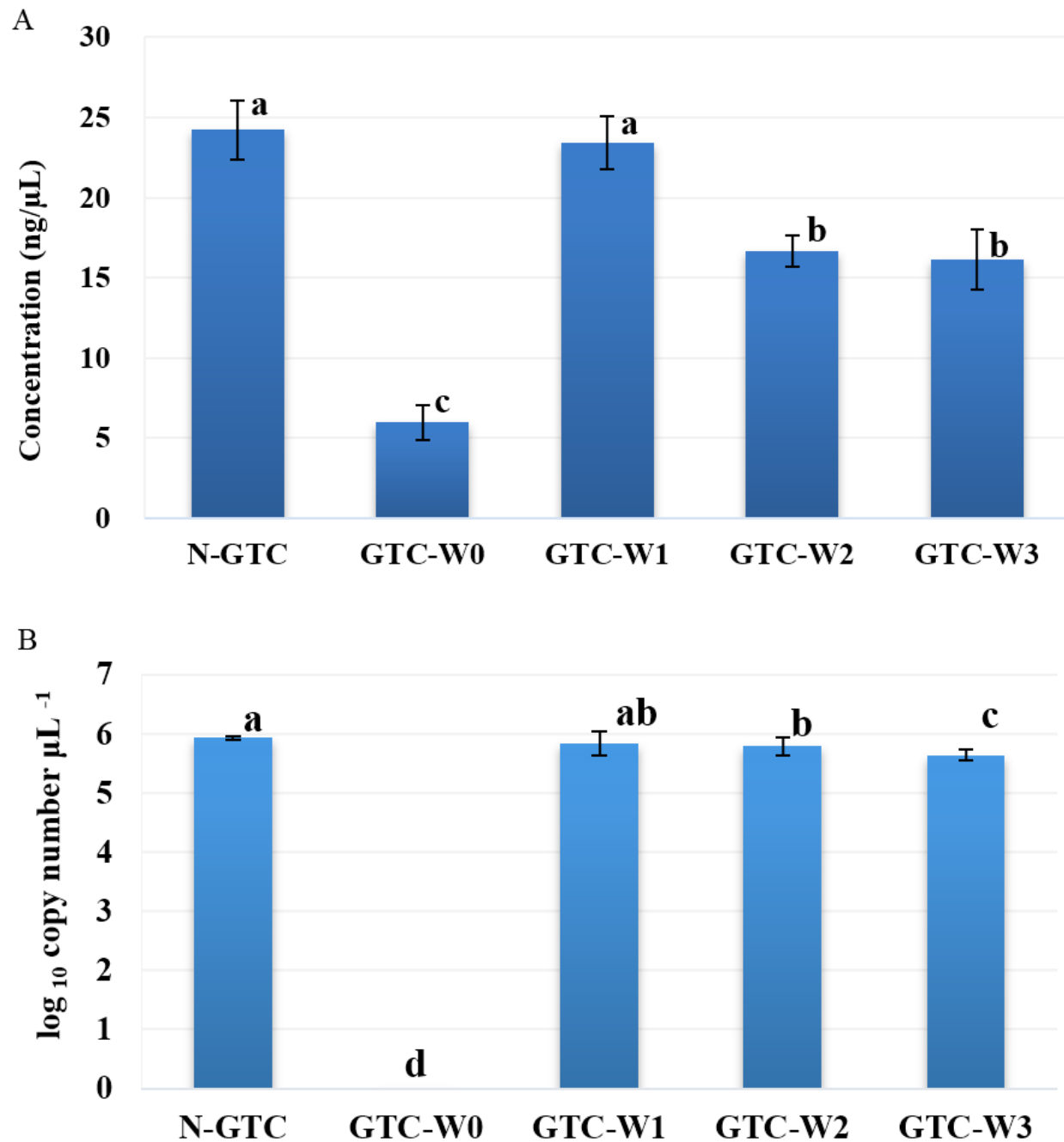



Figure 3

The distribution of top 15 genera in the FR, GTC, and RT groups (A). Comparisons of the taxonomic abundances of top 15 genera between FR and RT groups (B), and FR and GTC groups (C)

FR-2d, rhizosphere samples stored at -70°C for 2 days (n = 3). GTC-2d, -8d, and 31d are samples stored in 100 μL of GTC guanidine thiocyanate at room temperature for 24 h, and -20° for 1, 7, and 30 days, respectively (n = 3). RT-2d, -8d, and 31d are samples stored at room temperature for 2, 8, and 31 days, respectively (n = 3). The error bars represent standard deviation.

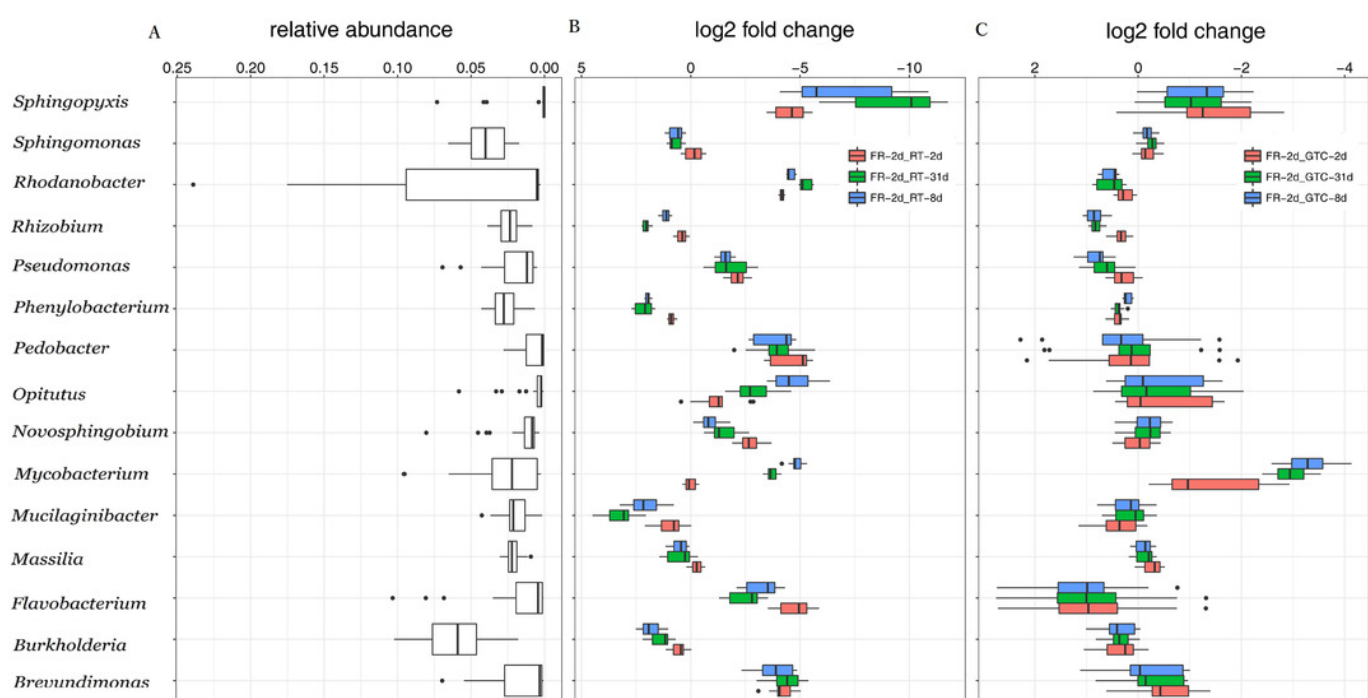



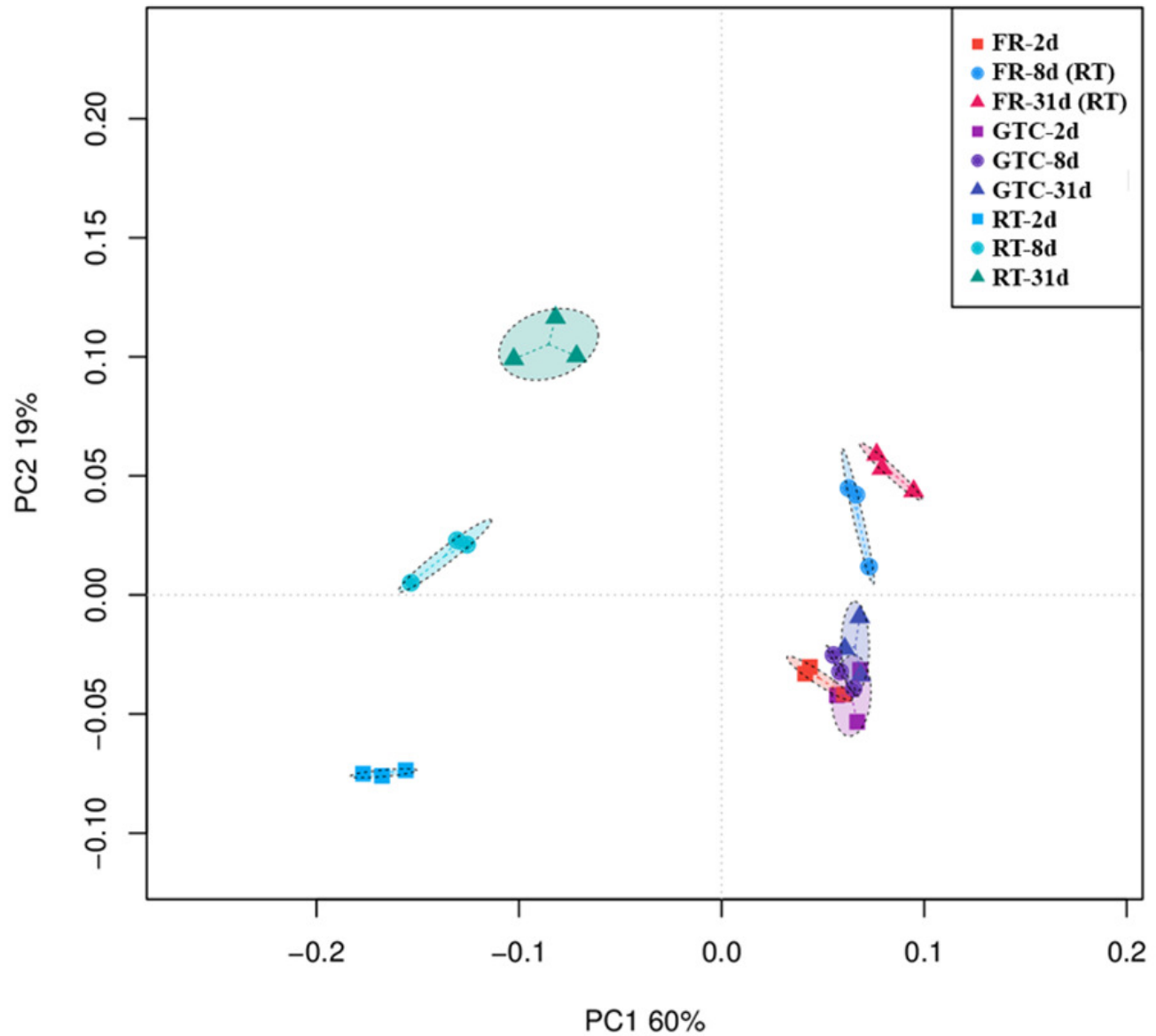
Figure 4

Relative abundance of Pseudomonas spp. in all rhizosphere samples of peanuts, as determined by real-time qPCR and pyrosequencing.

FR-2d, rhizosphere samples stored at -70°C for 2 days (n = 3). FR-8d (RT) and FR-31d (RT) are rhizosphere samples stored at -70°C for 8 and 31 days, respectively, thawed at room temperature for 15 min before DNA isolation (n = 3). GTC-2d, -8d, and 31d are samples stored in 100 µL of GTC guanidine thiocyanate at room temperature for 24 h, and -20° for 1, 7, and 30 days, respectively (n = 3). RT-2d, -8d, and 31d are samples stored at room temperature for 2, 8, and 31 days, respectively (n = 3). The error bars represent standard deviation. [a,b,c] Different lower-case letters indicate significant differences between groups analyzed by one-way ANOVA (Tukey's HSD) analysis ($p < 0.05$).

Figure 5

Weighted UniFrac principal coordinate analysis (PCoA) of the rhizosphere bacterial community structure following the different treatments.

FR-2d, rhizosphere samples stored at -70°C for 2 days (n = 3). FR-8d (RT) and FR-31d (RT) are rhizosphere samples stored at -70°C for 8 and 31 days, respectively, thawed at room temperature for 15 min before DNA isolation (n = 3). GTC-2d, -8d, and 31d are samples stored in 100 μL of GTC guanidine thiocyanate at room temperature for 24 h, and -20° for 1, 7, and 30 days, respectively (n = 3). RT-2d, -8d, and 31d are samples stored at room temperature for 2, 8, and 31 days, respectively (n = 3). Ellipses, 95% confidence intervals.

Pyrosequencing of 16S rRNA and alpha-diversity indices

*Three independent biological replicates were analyzed in each group. Different lower-case letters indicate significant differences between groups analyzed by one-way ANOVA (LSD) analysis ($p < 0.05$).

2 **Table 1** Pyrosequencing of 16S rRNA and alpha-diversity indices

Treatment[*]	High-quality sequences	OTU number	Chao index	Shannon index	SRA database accession no.
FR-2d	56214.0±6924.0b	2066.0±155.0b	2068.2±154.7b	8.3±0.1b	
RT-2d	58342.0±399.0ab	1347.0±63.0c	1350.1±63.5c	7.0±0.1d	
GTC-2d	60940.0±863.0ab	2314.0±37.0a	2316.0±37.1a	8.5±0.0a	
FR-8d (RT)	58259.0±4254.0ab	2219.0±44.0ab	2221.0±44.0ab	8.5±0.1ab	
RT-8d	62145.0±646.0a	1446.0±63.0c	1450.3±62.7c	7.2±0.2c	SRP134256
GTC-8d	60640.0±273.0ab	2236.0±48.0ab	2237.4±48.4ab	8.4±0.1ab	
FR-31d (RT)	60987.0±1351.0ab	2176.0±18.0b	2178.0±17.9b	8.5±0.1ab	
RT-31d	62335.0±322.0a	1192.0±68.0d	1195.9±67.6d	6.9±0.1d	
GTC-31d	61742.0±1489.0a	2302.0±24.0a	2303.6±24.4a	8.5±0.0a	

3 [*] Three independent biological replicates were analyzed in each group. Different lower-case letters
4 indicate significant differences between groups analyzed by one-way ANOVA (LSD) analysis (p
5 < 0.05).