

QTL Analysis for Soybean (Glycine Max L. Merr.) Seed Storability in High-Temperature Storage Conditions

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1 **QTL analysis for soybean (*Glycine max* L. Merr.) seed storability in high-temperature storage**
2 **conditions**

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31 **ABSTRACT**

32 Soybean (*Glycine max* L. Merr.) seeds are highly sensitive to the environmental conditions experienced
33 in storage. The objectives of this study were to identify QTLs for soybean seed storability by evaluating
34 seed viability and seed vigor and investigating candidate genes for these traits in detected QTL regions.
35 These objectives will aid in developing soybean cultivars with a high capacity for storability. The seeds
36 of 109 and 90 recombinant inbred lines (RILs) derived from a cross between Misuzudaizu and Moshidou
37 Gong 503 in 2010 and 2019, respectively, were used to evaluate seed viability by a germination test and
38 seed vigor by an accelerated aging test, after storage under two temperature conditions (25 °C and 35 °C)
39 for six months. Seed viability and seed vigor of Moshidou Gong 503 were higher than those of
40 Misuzudaizu in both temperature conditions. The average seed viability and seed vigor of RILs decreased
41 when stored at the higher temperature. A total of five QTLs were found for the two traits, seed viability
42 and seed vigor, located in chromosomes A2, C2, and D1b. The Misuzudaizu alleles decreased seed
43 viability and seed vigor at all detected QTLs. Most QTLs in this study were found near loci controlling
44 seed viability, maturity, germination, seed hardness, and seed surface micromorphology, indicating that
45 seed storability is related to these traits. In addition, two new QTLs were found that are associated with
46 seed storability.

47

48 Keywords: quantitative trait locus (QTL), seed viability, seed vigor, soybean seed storability, storage
49 condition

50 INTRODUCTION

51 Seed storability is of paramount importance in agricultural production. Short longevity due to
52 loss in seed viability and vigor during storage negatively impacts seedling development and crop
53 productivity (Han et al. 2014; Börner et al. 2018). Reduced seed viability and seed vigor due to seed
54 deterioration is influenced by high temperature and humidity in the storage environment that affects
55 biochemical processes in seeds, such as increases in hydrolytic enzyme activity, enhanced respiration,
56 and lipid peroxidation leading to increased levels of free fatty acids (Copeland and McDonald 2001).
57 Lipid peroxidation (Wang et al. 2018) and protein damage (Davies 2005) always occur in oilseed crops
58 during high-temperature storage. In humid tropical and sub-tropical climates, including Southeast Asia,
59 where temperature and relative humidity are high, the rapid loss in seed germination ability during
60 storage is a major problem in soybean production (Nkang et al. 1996).

61 Soybean is the most important legume crop globally because the seeds contain large amounts
62 of storage components, including proteins and lipids, used for food, feed and many industrial materials.
63 Seed proteins and lipids are susceptible to high temperatures and elevated humidity in storage. In
64 unconditioned storage environments, soybean seed deterioration occurs rapidly (TeKrony et al. 1993).
65 Lengthy storage of soybeans causes reduced seed longevity, lower levels of germination, and impaired
66 seedling establishment due to the utilization of several molecules during seed respiration. Thus,
67 developing cultivars with significant resistance to high-temperature storage is necessary to improve seed
68 viability and vigor during storage. Nevertheless, understanding the genetic bases for seed storability is
69 insufficient; we must also develop genetic material with resistance to storage conditions. Genetic
70 analyses should be conducted to identify soybean genotypes that are resistant to storage conditions and
71 to develop genetic markers for marker-assisted selection (MAS).

72 Several genetic analyses of seed storability have been reported in *Arabidopsis* (Clerkx et al.
73 2004), rice (Sasaki et al. 2005; Xue et al. 2008; Wang et al. 2010 Li et al. 2012; Miura et al. 2002), rye
74 seed (Chwedorzewska et al. 2002), safflower (Vijay et al. 2009) and soybean (Green and Pinnell 1968;
75 Lanyon 1970; Kueneman 1983; Singh and Ram 1986; Verma and Ram 1987; Cho and Scott 2000; Singh
76 et al. 2008). Verma and Ram (1987) found that two to four genes presumably control seed longevity in
77 soybean. Polymorphisms in RAPD profiles of aged soybean seeds were found and four SSR markers
78 were associated with seed coat permeability and electrolyte leaching in soybean (Singh et al. 2008). Four
79 SSR markers related to seed longevity in an F_{2:3} soybean population derived from a cross between good

80 and poor storage genotypes have been reported (Singh et al. 2008a). Vijay et al. (2009) clustered the
81 genetic variation of safflower and soybean into three different seed age groups, including un-aged,
82 naturally aged, and age-accelerated seeds by RAPD, AFLP, and SSR markers. RAPD markers clustered
83 naturally aged and age-accelerated seeds types in soybean and safflower, whereas the control and
84 naturally aged seed were clustered by SSR and AFLP markers (Vijay et al. 2009).

85 Quantitative Trait Loci (QTL) analysis is effective because seed storability is a quantitative trait
86 controlled by several genes. Several QTL analyses of seed storability have been reported in Arabidopsis
87 (Bentsink et al. 2000; Clercx et al. 2004), rice (Sasaki et al., 2005; Xue et al. 2008; Wang et al. 2010; Li
88 et al. 2012; Miura et al. 2002), barley (Nagel et al. 2016), maize (Han et al. 2014) and oilseed rape (Nagel
89 et al. 2011). In soybean, QTLs associated with seed storability remain to be identified and characterized,
90 and there are no studies of QTL analysis for seed storability based on seed vigor and under high
91 temperature and long term storage condition. Although QTLs controlling seed storability have been
92 reported, genes responsible for seed storability have not yet been identified. Dargahi et al. (2014) reported
93 that there are three QTLs on three linkage groups that are associated with seed storability. However, this
94 report found the QTLs in seeds under ambient condition and accelerated aging.

95 In this study, a RIL population derived from a cross between Misuzudaizu and Moshidou Gong
96 503 was stored under two different temperatures to evaluate seed viability and seed vigor. The objectives
97 of this study were to identify QTLs for seed storability at the two different temperatures and analyze the
98 responsible genes by detecting QTL regions.

99

100 **MATERIALS AND METHODS**

101 **Plant materials**

102 Recombinant inbred lines (RILs) derived from a cross between Misuzudaizu and Moshidou
103 gong 503 were obtained from the National BioResource Project (NBRP) for *Lotus* and *Glycine*,
104 University of Miyazaki. Misuzudaizu is a yellow soybean seed cultivar developed at the Nagano
105 Prefectural Agricultural Experiment Station, Japan and Moshidou Gong 503 is a brown soybean seed
106 line originating from Jilin province, China. In 2010 and 2019, 109 and 90 RILs, respectively, were used
107 in this study.

108 **Seed storage conditions**

109 Seeds were stored in paper bags (19.5 cm × 11.9 cm) at 25±1 °C or 35±1 °C at 40–57% relative
110 humidity for six months.

111 **Seed viability**

112 Seed viability was evaluated by the between-paper germination test (Rao et al. 2006). Three
113 replications of 30 seeds from the RILs were placed between moistened double-folded paper towels (size
114 40 × 33 cm). After being scarified to break seed dormancy, the seeds were moved to Plant Boxes for
115 Plant Culture (125mL, size 75 × 75 × 100 mm VWR International, USA) and placed into incubators at
116 25 °C for eight days (ISTA 2011). At the end of the treatment, the number of normal seedlings was
117 counted. The germination percentage was calculated as the number of normal seedlings/number of all
118 seeds × 100.

119 **Seed vigor**

120 The accelerated aging (AA) test (ISTA 2011) was used to evaluate seed vigor. Seeds were
121 treated at 41 ± 1 °C and 100% relative humidity for 72 h. Subsequently, the between-paper germination
122 test was conducted. After eight days, the number of normal seedlings was counted. Seeds with higher
123 germination percentages indicated greater seed vigor.

124 **QTL analysis**

125 The markers used in this study were a total of 1,131 simple sequence repeat (SSR) markers from
126 the Glycine max / G. soja database at LegumeBase:
127 (<https://www.legumebase.brc.miyazakiu.ac.jp/glycine/riChromosome/riChromosomeList.jsp>) where
128 genotypes and physical maps of all RIL populations are also available. The map had a distance of
129 3,240.72 cM among 20 linkage groups (LGs), and the average genetic distance between two neighboring
130 marker loci was 2.87 cM. The presence of QTLs was calculated by composite interval mapping using
131 R/qtl software (Broman et al. 2003). The logarithm of odd (LOD) value significance threshold was
132 determined by a permutation test module (1000 replications).

133 **Data analysis**

134 The effects of genotype and storage temperature on seed viability and seed vigor were indicated
135 by seed germination and AA tests, based on an analysis of variance (ANOVA), calculated by R software.
136 All treatments were considered as effects, whereas interactions with replication were treated as random
137 effects. Means and interactions were compared by Duncan's test with least-square means comparisons.

138

139 **RESULTS**

140 **Seed viability**

141 The initial germination percentages of Misuzudaizu in 2010 and 2019 were 80% and 60%,
142 respectively, whereas those of Moshidou Gong 503 were 80% and 100%, respectively. In contrast, the
143 initial germination percentage of the RILs in 2010 ranged from 0% to 100% with an average of 59.75% %,
144 and those of the RILs in 2019 ranged from 0% to 90% with an average of 26.33% (Table 1). The
145 germination percentages of Misuzudaizu 503 in 2010 and 2019 after storage at 25 °C for six months were
146 10% and, 60%, respectively, and those of Moshidou Gong 503 were 45% and, 80%, respectively. The
147 germination percentage of the RILs stored at 25 °C for six months in 2010 ranged from 0% to 73% with
148 an average of 29.57%, and those of the RILs in 2019 ranged from 0% to 100% with an average of 34.33%
149 (Table 1). Storage at 35 °C for six months resulted in a germination percentage for Misuzudaizu in 2010
150 and 2019 of 0% and 40%, respectively and those of Moshidou Gong 503 were 20%, and 60%,
151 respectively. The 2010 RILs had germination percentages ranging from 0% to 60% with an average of
152 7.19% (Figure 1.), and those of the 2019 RILs ranged from 0% to 100% with an average of 23.67%
153 (Table 1).

154 **Seed vigor**

155 After conducting the accelerated aging test, the germination percentages of Misuzudaizu in
156 2010 and 2019 were 10% and 20%, respectively, and those of Moshidou Gong 503 were 40%, and 80%,
157 respectively. Germination percentages of the 2010 RILs ranged from 0% to 80% with an average of
158 15.63%, and those of the 2019 RILs ranged from 0% to 80% with an average of 12.56% (Table 1, Figure
159 1). The germination percentages of Misuzudaizu stored at 25 °C for 6 months in 2010 and 2019 were 0%
160 and 40%, respectively, whereas those of Moshidou Gong 503 were 20%, and 80%, respectively. In
161 contrast, the germination percentages of the 2010 RILs ranged from 0% to 60% with an average of 4.59%,
162 and those of the 2019 RILs ranged from 0% to 80% with an average of 15.89% (Table 1, Figure 1.). The
163 germination percentages of Misuzudaizu 503 in 2010 and 2019 stored at 35 °C for 6 months were 0%
164 and 20%, respectively, whereas those of Moshidou Gong 503 were 10% and 60%, respectively. RILs
165 stored at 35 °C for 6 months in 2010 ranged from 0% to 60% with an average of 3.85%, and similarly
166 stored RILs in 2019 ranged from 0% to 90% with an average of 11.54% (Table 1).

167 **Effect of line and storage temperature on seed viability and seed vigor**

168 An analysis of variance for seed viability and seed vigor after storage at each condition is
169 shown in Table 2. Significant two-way interactions among lines X storage temperature ($P>0.0001$) were
170 observed for seed viability and seed vigor, except for seed viability in 2019.

171

172 **QTL analysis**

173 A total of five QTLs were identified for two traits, seed viability and seed vigor (Table 3). For
174 seed viability after storage at 35 °C for six months, a QTL (*qSG-A2*) with an LOD value of 4.03 and a
175 phenotypic variance of 6.84% was detected on Chromosome A2. In seeds tested for seed vigor in the
176 untreated condition in 2010, a QTL (*qSV-C2.1*) was detected on Chromosome C2 with an LOD value of
177 3.64 and a phenotypic variance of 16.6%. For seed vigor at 35 °C for six months, a QTL (*qSV-C2.2*) was
178 detected on Chromosome C2 in 2010 with an LOD value of 2.95 and a phenotypic variance of 4.34%.
179 For seed vigor in the untreated condition in 2019, a QTL (*qSV-C2.3*) was detected on Chromosome C2
180 with an LOD value of 4.13 and a phenotypic variance of 13.1%. For seed vigor at 25 °C for six months,
181 a QTL (*qSV-D1b*) was detected on Chromosome D1b with an LOD value of 5.02 and a phenotypic
182 variance of 16.6% (Figure 2).

183

184 **DISCUSSION**

185 Soybean is categorized in the poor storability group of crop plants (Justice and Bass 1978). The
186 environmental conditions for storage are significant factors influencing seed viability and vigor. This
187 study used soybean recombinant inbred lines derived from a cross between Misuzudaizu (yellow) and
188 Moshidou Gong 503 (brown) harvested in 2010 and 2019 and used to evaluate seed viability and vigor
189 and to identify QTLs controlling seed storability. In comparing the germination percentage in the parental
190 lines, Moshidou Gong 503 had a higher germination percentage in high temperature and long-term
191 storage than Misuzudaizu. After conducting the AA test, the germination percentage of Misuzudaizu,
192 Moshidou Gong and the RILs decreased (Table 1). In particular, the germination percentage of
193 Misuzudaizu was as low as 10% in untreated seed, whereas AA treated seeds failed to germinate in 2010
194 (Table 1).

195 Genetic factors play a role in soybean seed storability (Shelar et al. 2008). Seed storability is a
196 complex quantitative trait controlled by multiple genes and is easily influenced by the environment.
197 Genetic loci associated with seed storability should be identified to develop cultivars resistant to

198 storability issues and to characterize ways of controlling seed storability. This study found five QTLs
199 associated with seed storability, four QTLs for seed vigor and one QTL for seed viability. A Misuzudaizu
200 allele decreased seed viability at *qSG-A2* and showed similar effects at the QTLs *qSV-C2.1*, *qSV-C2.2*,
201 *qSV-C2.3* and *qSV-D1b.1*. The QTLs *qSV-C2.1*, *qSV-C2.2* and *qSV-C2.3* were located and closely placed
202 on the same chromosome C2. Only *qSV-D1b.1* was located elsewhere on chromosome D1b. Watanabe
203 et al. (2004) found sixty-six QTLs associated with three reproductive development traits and four seed
204 quality traits as well as Otobe et al. (2015) cloud detected five QTLs associated with micromorphology
205 on the seed coat surface in RILs population derived from a cross between Misuzudaizu and Moshidou
206 Gong 503 using the total of 360 markers. However, the total of 1,131 markers were used in this study
207 and five QTLs associated with seed storability were detected. In comparing our QTL results with QTLs
208 for seed quality, we found that the *qSV-C2.2* and *qSV-C2.3* QTLs are new because they have not been
209 associated with any other QTLs in previous reports. The *qSG-A2* QTL was located close to QTLs for
210 maturity (*HAV4*), seed viability (*VIS4*) (Watanabe et al. 2004), and seed surface micromorphology
211 (Otobe et al. 2015). The mapped location of *qSV-C2.1* was close to QTLs for germination rate (*GRS1*)
212 and seed hardness (*RAS1*) (Watanabe et al. 2004). The *qSV-D1b.1* QTL was located close to QTLs for
213 seed hardness (*RAS2*) (Watanabe et al. 2004) and a seed surface trait (*qSR3*) (Otobe et al. 2015). For
214 QTL associated with seed storability, Daragahi et al. (2004) used the total of 128 SSR makers constructed
215 38 linkage groups and identified three QTLs on three linkage groups (C1, F and L) associated with seed
216 storability in soybean under ambient condition ($28 \pm 2^\circ\text{C}$) for 5 months and accelerated aging ($42 \pm 1^\circ\text{C}$)
217 for 72 h, and no stable QTL were found in different years. However, this study used 1,131 SSR markers
218 and found five QTLs associated with seed storability on three linkage groups (A2, C2 and D1b) from 20
219 linkage groups, three QTLs located on same linkage group (C2) and the QTLs for seed vigor of untreated
220 seeds in both 2010 and 2019 were stable QTLs. Moreover, QTLs for seeds viability under high
221 temperature condition (35°C for 6 months) were found in this study.

222 Our study identifies for the first time two new QTLs for seed vigor that were found in treated
223 seeds stored at 35°C for six months in 2010 and untreated seeds in 2019. Seed vigor analyzed by the
224 accelerated aging test (AA Test) imitates stressful conditions for soybean seeds. Seeds with high vigor
225 can tolerate stressful conditions during the AA Test. Therefore, evaluating seed vigor by the AA Test
226 can detect changes in vigor more sensitively than the standard germination test. For seed viability,
227 Watanabe et al. (2004) found five QTLs associated with seed viability; however, only *qSG-A2* was found

228 in our study since seeds were stored long term at a high temperature. Moreover, *qSG-A2* was found to be
229 located closely positioned on the same chromosome as *VIS4*, a QTL initially reported by Watanabe et al.
230 (2004).

231 Seed longevity is thought to be progressively acquired during maturation and reaches its
232 maximum during the seed filling phase or a later maturation phase (Zanakis et al. 1994; Gillen et al.
233 2012; Marcos-Filho J. 2016; Lima et al. 2017). The finding that the *qSG-A2* QTL was located close to
234 the *HAV4* QTL may indicate a relationship between seed longevity and seed maturation.

235 Pigmented seeds have higher germination rates and vigor than unpigmented seed coats and
236 exhibit higher resistance to deterioration during storage (Singh and Ram 1986; Liu et al. 2017). Most
237 hard seed characteristics in dark-colored soybeans are associated with permeability and protection
238 against abiotic and biotic stresses and function to protect seeds from deterioration and to maintain seed
239 vigor during long storage periods. There are multiple cell layers in the seed coats of dark seeds filled
240 with phenolic compounds that function as antioxidants (Liu et al. 2017) and may act to reduce metabolic
241 activity. Moshidou Gong 503 is a brown accession with a high degree of seed storability due to increased
242 seed hardness and a smooth seed surface (Otobe et al. 2015). Smooth seed surfaces are derived from
243 waxy materials that protect the seed from the environment and confer seed impermeability. Most yellow
244 soybean seeds lose germination ability during storage and aging due to their thin and weak seed coats
245 with wide pores. Although seed dormancy was broken by scarification in our study, some seeds were not
246 viable and could not germinate. Soybean seeds stored long term in high temperature conditions had low
247 seed viability and seed vigor. During storage, the influence of fatty acid metabolism continues to be
248 important. During longer storage periods, the concentrations of fatty acid glucosides and aglycons
249 increase, whereas the levels of acetyl glucoside and malonyl glucoside decreased after three years (Liu
250 et al. 2017). Thus, deterioration at the biochemical, physiological and molecular levels may occur during
251 storage, especially lipid peroxidation and membrane lipid hydrolysis.

252 In conclusion, seed storability is related to several seed quality traits. Most QTLs controlling
253 seed storability are related to other QTLs for seed quality. The two new QTLs found in this study will
254 provide useful information for identifying genes responsible for seed storability and for developing
255 soybean cultivars with higher degrees of storability in the future. Although QTLs that regulate seed
256 storage have been discovered, seed storability depends on the biochemical and physiological processes
257 occurring in stored seeds. Changes in seed components that occur during seed storage are dynamic, and

258 the relationship between the degrading enzymes and other factors involved in these changes should be
259 analyzed in the future.

260

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263 Research and Development (AMED).

264

265

266 **Author contributions**

267 For conceptualization was design by MH, KH and PJ. Material preparation and data collection were
268 performed by PJ. Data analysis was performed by PJ and IL. The first draft of the manuscript was written
269 by PJ and MH and all author commented on previous versions of the manuscript. All authors read and
270 approved the final manuscript.

271

272 **Ethics declarations**

273 **Conflicts of interest** The author declare that there is no conflict of interest.

274 **Ethics approval** This research did not involve research with human participants and/or animals.

275 **Consent to participate** All author have reviewed the manuscript and approved their participation.

276 **Consent for publication** All author have reviewed the manuscript and approved its submission.

277

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280 Medical Research and Development (AMED).

281

282 **Data availability**

283 The dataset in this study is available on the on the *Glycine max* / *G. soja* database in LegumeBase:
284 (<https://www.legumebase.brc.miyazakiu.ac.jp/glycine/ri1Chromosome/ri1ChromosomeList.jsp>).

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383 **Fig 1.** Frequency distribution of seed viability and seed vigor in each storage condition for RIL seeds in
384 2010 and 2019. (A) seed viability of 35°C /6 months storage in 2010, (B) seed vigor of untreated seed
385 in 2010, (C) seed vigor of 35°C/6 months storage in 2010, (D) seed vigor of untreated seed in 2019 and
386 (E) seed vigor of 25°C/6 months storage in 2019. ∇: Misuzudaizu, ▼: Moshidou Gong 503

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388 **Fig 2.** Quantitative trait locus (QTL) analysis, showing composite interval mapping for seed viability
389 (*qSG*) and seed vigor (*qSV*) in 2010 (----) and 2019 (—), Black arrowheads indicate QTL for seed
390 viability and seed vigor.

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392 **Table 1** Analysis of seed viability and seed vigor in the parental and RIL populations

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Year	Storage condition	Accelerated aging (AA)*	Germination rate (%)			
			Parents		RIL population	
			Misuzudaizu	Moshidou Gong 503	Range	Average
2010	Untreated	-	80	80	0-100	59.75
		+	10	40	0- 80	15.63
	25 °C	-	10	45	0- 73	29.57
		+	0	20	0- 60	4.59
	35 °C	-	0	20	0- 60	7.19
		+	0	10	0- 60	3.85
2019	Untreated	-	60	100	0- 90	26.33
		+	20	80	0- 80	12.56
	25 °C	-	60	80	0-100	34.33
		+	40	80	0- 80	15.89
	35 °C	-	40	60	0-100	23.67
		+	20	60	0- 90	11.54

409 * Seeds were germinated after with (+) and without (-) an accelerated aging (AA) treatment.

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412 **Table 2** Analysis of variance for seed viability and seed vigor of 109 RILs in 2010 and 109 RILs in 2019 after storage at each condition.

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Effect	Seed viability						Seed vigor					
	2010			2019			2010			2019		
	df	F- value	P > F	df	F- value	P > F	df	F- value	P > F	df	F- value	P > F
Line (L)	108	4.30	<0.0001	89	3.34	<0.0001	108	15.20	<0.0001	89	7.04	<0.0001
Temperature (T)	2	725.27	<0.0001	2	5.14	0.0067	2	172.81	<0.0001	2	5.91	0.0032
L × T	216	2.82	<0.0001	178	1.07	0.3185	216	5.39	<0.0001	178	2.27	<0.0001

424 **Table 3.** Summary of detected QTLs for seed storability of RILs in 2010 and 2019

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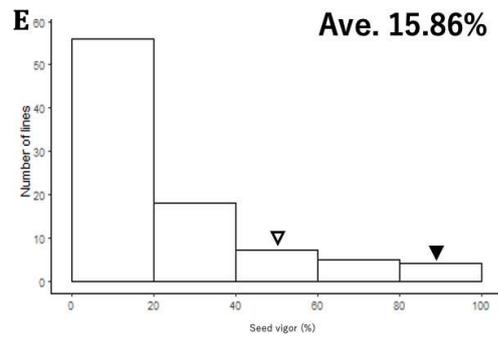
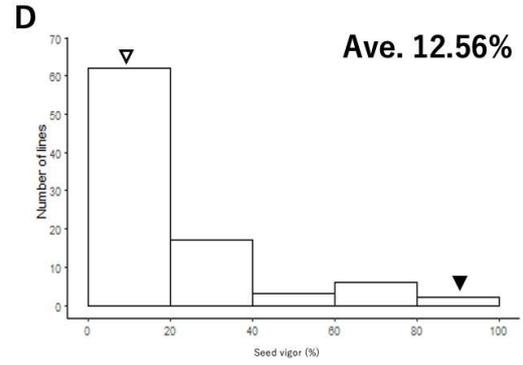
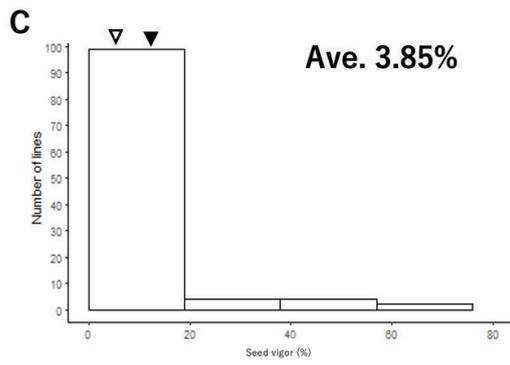
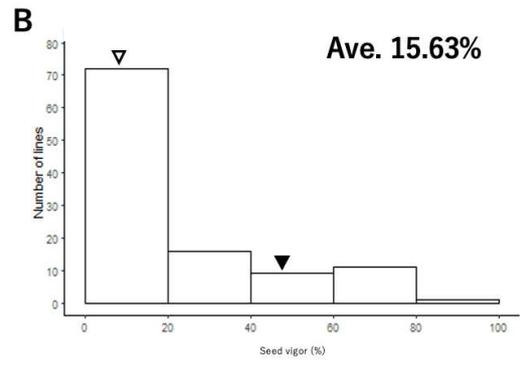
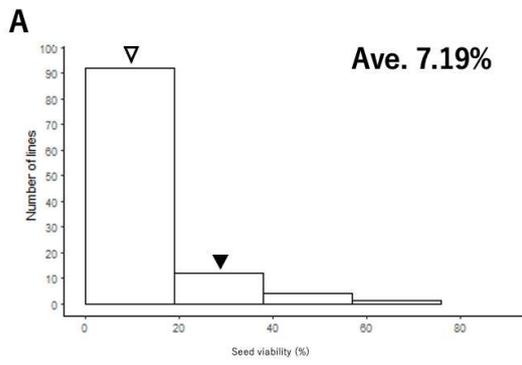
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Year	Traits	Condition	QTL	LG	Marker	Position (cM)	LOD	PVE	R ² (%)
2010	Seed viability	35°C/6 M	<i>qSG-A2</i>	A2	GMES1620	53	4.03	-5.13	6.84
	Seed vigor	Untreated	<i>qSV-C2.1</i>	C2	MOJI.C	119	3.64	-8.31	16.6
		35°C/6 M	<i>qSV-C2.2</i>	C2	E8M18Mo1000A	121.9	2.95	-4.07	4.34
2019	Seed vigor	Untreated	<i>qSV-C2.3</i>	C2	Sat_238	128	4.13	-9.79	13.1
		25°C/6 M	<i>qSV-D1b</i>	D1b	GMS05538	127	5.02	-11.4	16.6

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