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To cite this article: Atsuko Miyagi, Ko Noguchi, Takeshi Tokida, Yasuhiro Usui, Hirofumi Nakamura, Hidemitsu Sakai, Toshihiro Hasegawa & Maki Kawai-Yamada (2019) Oxalate contents in leaves of two rice cultivars grown at a free-air CO₂ enrichment (FACE) site, *Plant Production Science*, 22:3, 407-411, DOI: [10.1080/1343943X.2019.1598272](https://doi.org/10.1080/1343943X.2019.1598272)

To link to this article: <https://doi.org/10.1080/1343943X.2019.1598272>



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Published online: 05 Apr 2019.



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SHORT REPORT



Oxalate contents in leaves of two rice cultivars grown at a free-air CO₂ enrichment (FACE) site

Atsuko Miyagi^a, Ko Noguchi^b, Takeshi Tokida^c, Yasuhiro Usui^d, Hirofumi Nakamura^e, Hidemitsu Sakai^f, Toshihiro Hasegawa^g and Maki Kawai-Yamada^a

^aGraduate School of Science and Engineering, Saitama University, Saitama, Japan; ^bSchool of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan; ^cDivision of Biogeochemical Cycles, Institute for Agro-Environmental Sciences, Ibaraki, Japan; ^dDivision of Farming System Research, Hokkaido Agricultural Research Center, Memuro, Hokkaido, Japan; ^eTaiyo Keiki Co. Ltd., Tokyo, Japan; ^fDivision of Climate Change, Institute for Agro-Environmental Sciences, Ibaraki, Japan; ^gDivision of Agro-Environmental Research, Tohoku Agricultural Research Center, Morioka, Iwate, Japan

ABSTRACT

Rice straw can be used as a feed for livestock. It accumulates soluble oxalate, which is harmful to livestock as it can cause mineral deficiency. It is therefore necessary to reduce the oxalate content in rice leaves so that the usage of rice straw for a livestock feed can be increased. Several oxalate synthesis pathways have been reported in various plants. However, the oxalate synthesis pathway in rice has not yet been elucidated. In the present study, we found that the oxalate content of the leaves of two rice cultivars, Koshihikari and Takanari, was different. The differences in the oxalate contents in the leaves of two cultivars were also maintained under the elevated CO₂ concentration and/or temperature conditions. These findings suggest that oxalate accumulation has a genetic basis and does not depend on CO₂ and temperature conditions.

Abbreviations: A-[CO₂]: ambient CO₂ concentration; CE-QQQ-MS: capillary electrophoresis-triple quadrupole-mass spectrometry; E-[CO₂]: elevated CO₂ concentration; E-T: elevated soil/water temperature; FACE: free-air CO₂ enrichment; TCA: tricarboxylic acid; N-T: normal soil/water temperature

ARTICLE HISTORY

Received 10 September 2018
Revised 6 March 2019
Accepted 7 March 2019

KEYWORDS

Oryza sativa; Koshihikari;
Takanari; oxalate;
CE-QQQ-MS

Rice (*Oryza sativa* L.) is not only an important food crop for humans, but it is also used as a feed for livestock. Rice straw is composed of leaves and stems and accumulates higher levels of soluble oxalate than other poaceous species (Jackson, 1978). In some plant species, oxalate, which is the simplest dicarboxylic acid, is stored in the leaves and acts as a chemical defence substance against predators (Franceschi and Nakata, 2005), detoxifies Al³⁺ in acidic soils (Ma, Ryan & Delhaize, 2001; Miyagi, Uchimiya, Kawai-Yamada & Uchimiya, 2013a), and produces H₂O₂ which acts as a signalling molecule during wounding or aging (Davoine et al., 2001; Le Deunff, Davoine, Le Dantec, Billard & Huault, 2004). One of the chemical characteristics of oxalate is its high binding affinity with divalent inorganic cations, such as calcium, ferric and zinc ions. Because the binding of oxalate and divalent inorganic cations forms insoluble compounds, the excessive intake of rice straw by livestock can inhibit mineral absorption. Low oxalate content of rice leaves is therefore an important trait for the quality of rice straw as livestock feed.

Atmospheric CO₂ concentrations ([CO₂]) are rising at an unprecedented rate and are projected to increase further to drive global environmental changes such as global warming (IPCC, 2013). On the other hand, increasing [CO₂] is known to enhance crop photosynthesis and to affect the primary metabolism (Noguchi et al., 2018; Onda, Miyagi, Takahara, Uchimiya & Kawai-Yamada, 2014), but its effect on oxalate biosynthesis in rice is unknown. In plants, several oxalate biosynthesis pathways have been identified (Franceschi and Nakata, 2005; Millerd, Morton & Wells, 1963a, 1963b; Miyagi, Uchimiya, Kawai-Yamada & Uchimiya, 2013b; Richardson & Tolbert, 1961; Seal & Sen, 1970), including the glycolate pathway (glycolate is oxidised to oxalate via glyoxylate by glycolate oxidase in the photorespiration pathway), the isocitrate pathway (isocitrate is converted to glyoxylate and succinate by isocitrate lyase in the glyoxylate cycle), and the ascorbate pathway (ascorbate is converted to oxalate via several steps). However, which of these pathways contributes to oxalate accumulation in rice leaves remains unknown, particularly in response to change in [CO₂].

In our previous study, dynamic changes of the primary metabolite contents were tested for two rice cultivars, cv. Takanari and cv. Koshihikari, cultivated at free-air CO₂ enrichment (FACE) site (Noguchi et al., 2018). The two cultivars showed distinct levels of some primary metabolites, but oxalate contents has not been reported. Thus, we measured oxalate content of the samples and found that the oxalate content in leaves of Takanari was lower than in Koshihikari in the present study. To clarify whether the differences in oxalate contents depend on environmental factors, such as elevated CO₂ concentrations (E-[CO₂]) or temperature (E-T) changes, oxalate contents in rice grown at FACE site were measured by capillary electrophoresis-triple quadrupole-mass spectrometry (CE-QQQ-MS).

Materials and methods

Oxalate was measured in the plant samples described previously (Noguchi et al., 2018). Details of the plant culture and sampling methods are given in Nakamura et al. (2012), Hasegawa et al. (2013), and Noguchi et al. (2018). Briefly, seeds of *Oryza sativa* L. cv. *Koshihikari* (*japonica* cultivar) and cv. *Takanari* (*indica* cultivar) were soaked and germinated. Three-week-old seedlings of these plants were transplanted at the Tsukuba FACE experimental facility in Tsukubamirai, Ibaraki, Japan (35°58'N, 139°60'E, 10 m a.s.l.) on May 23–24, 2013. Leaves of both cultivars were collected at the booting stage (July 22–25, 2013) and frozen immediately in liquid nitrogen. Four control plots (ambient CO₂ concentration (A-[CO₂]): 384 ± 11.4 μmol mol⁻¹) and four FACE plots (E-[CO₂]: 576 ± 15.5 μmol mol⁻¹) were used in this study. Normal temperature (N-T) and elevated temperature (E-T) (1.97 ± 0.08°C above N-T by placing heating wires on the soil surface between the rows) conditions were set up in both the control and FACE plots as split-plots. The details of the experimental setup, performance of [CO₂] and temperature control, and soil chemical properties were described previously (Adachi et al., 2014; Hasegawa et al., 2013; Nakamura et al., 2012; Usui et al., 2016). Each frozen leaf (approximately 50 mg) was ground and homogenized in liquid nitrogen and then added 50% methanol containing 50 μM PIPES and 50 μM methionine sulfone (as internal standards). After the first centrifugation step (22,000 g, 5 min, 4°C), the supernatant was transferred to a 3 kDa cut-off filter (Millipore, Billerica, MA, USA) and recentrifuged (14,000 g, 30 min, 4°C).

The oxalate content of this filtrate was then quantified by CE-QQQ-MS (CE; 7100, MS; 6420 Triple Quad LC/MS, Agilent Technologies, Santa Clara, CA, USA) in multi reaction monitoring (MRM) mode (Miyagi et al., 2010; Noguchi et al., 2018). A DB-WAX capillary (polyethylene

glycol-coated, 100 cm x 50 μm i.d., Agilent Technology) with 20 mM ammonium acetate (pH 8.5) was used as running buffer. MS analysis at the applied -25 kV was performed in negative ion mode. For MS stabilisation, 5 mM ammonium acetate (for anions) in 50% (v/v) methanol was used as a sheath solution, which was applied to the capillary at 10 μl min⁻¹ using an isocratic HPLC pump (Agilent 1200 series) equipped with a 1:100 splitter. The capillary voltage (-3500 V) and the drying nitrogen gas (at 320°C) flow (8 l min⁻¹) were kept constant for approximately 25 min during each electrophoresis run. Quantitative accuracy was determined using known concentrations of standard reference compounds using Agilent MassHunter Software.

Tests of statistical significance were carried out by a mixed model of SAS ('proc mixed' ver. 9.4, SAS Institute Inc., Cary, NC, USA). Our experimental design corresponded to a split-split-plot where [CO₂] was the main-plot factor, soil/water temperature the split-plot factor, and rice variety the split-split factor, with four replications. Variance components were estimated by the restricted maximum likelihood method with 'nobound' option (Littell, Milliken, Stroup, Wolfinger & Schabenberger, 2006). Using the metabolite data set described in the previous study (Noguchi et al., 2018), bivariate correlation analysis between oxalate and other metabolites was performed based on the Pearson's correlation coefficient as described in Miyagi et al. (2013c) using the IBM SPSS software package (v22.0 IBM, NY, USA).

Results & discussion

To compare the oxalate contents of leaves in Koshihikari and Takanari cultivated in A-[CO₂] condition, oxalate was measured by CE-QQQ-MS. As shown in Figure 1, the oxalate content in Takanari was approximately 5% of that in Koshihikari (Figure 1A and E). Takanari is a high-yielding *indica* cultivar, which exhibits semi-dwarf characteristics. The high-yields in Takanari can be attributed to the high spikelet number per panicle, which is associated with the high photosynthesis rate, high nitrogen accumulation, and a high carbohydrate translocation capacity during reproductive phase (Ohsumi et al., 2007; Takai et al., 2014, 2006). Generally, net nitrogen assimilation or nitrogen reductase activity is positively correlated with oxalate content (Miyagi et al., 2010, 2013b; Tian et al., 2008). Metabolomic analysis using CE-MS in a previous study showed that Takanari leaves accumulated more amino acids than Koshihikari leaves, although the organic acid contents of Takanari leaves were lower (Noguchi et al., 2018). Consequently, we expected that the oxalate content of Takanari leaves would be higher than that of Koshihikari

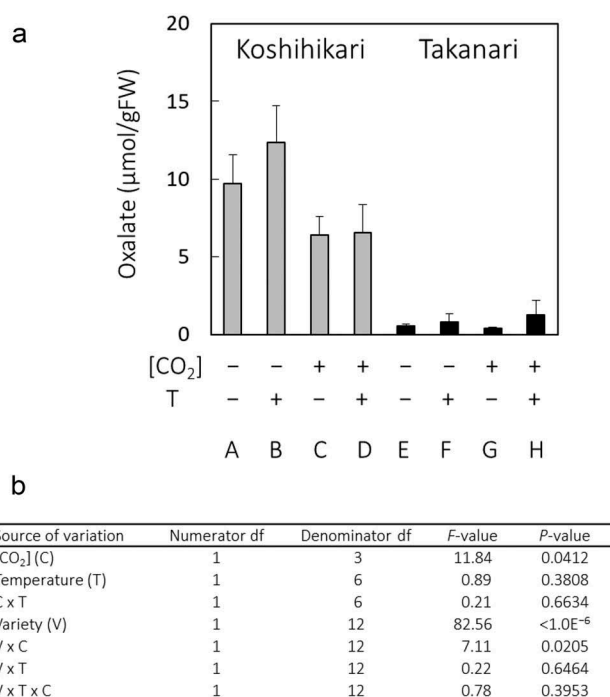


Figure 1. Oxalate content in leaves of three-month old rice grown at FACE site in Tsukubamirai-city, 2013 (a). [CO₂]; CO₂ concentration, T; soil/water temperature, n = 4, Bar; S.E. Statistical significance by a mixed model of SAS represents in (b).

leaves. However, the opposite result was observed in the present study, suggesting that the fixed carbon source in Takanari leaves was not used for oxalate synthesis but instead for amino acid accumulation.

Next, we investigated whether the oxalate content of Koshihikari and Takanari cultivated at FACE site was dependent upon the CO₂ concentration ([CO₂]) in the atmosphere. Specifically, we measured the oxalate content in the leaves of Koshihikari and Takanari at E-[CO₂]. The result showed that the difference in oxalate content of Koshihikari and Takanari leaves was maintained (Figure 1A, C, E, and G), although the oxalate content of Koshihikari leaves decreased slightly at high CO₂ levels. The decrease in oxalate content by the high [CO₂] condition was observed in other rice cultivars (Onda et al., 2014; Yu et al., 2010). The increase in [CO₂] suppresses the photorespiration pathway due to an increase in the carboxylation activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Thus, the observed decrease in oxalate content of Koshihikari leaves would likely have resulted from a decrease in glycolate content caused by the suppression of photorespiration. On the other hand, it has been reported that oxalate accumulation is independent of glycolate oxidase activity in rice (Xu et al., 2006); but it has also been reported that glyoxylate contributes

more to oxalate accumulation than ascorbate in rice (Yu et al., 2010). The differences between the present and previous studies might be attributed to differences in the [CO₂] and nitrate/ammonium balance in the soil. Moreover, to investigate the effects of E-T in soil and water on the oxalate contents of leaves under A-[CO₂] or E-[CO₂], the oxalate content in the leaves of Koshihikari and Takanari plants cultivated at the E-T (2°C above N-T) were measured. The oxalate content in both Koshihikari and Takanari leaves was not affected by E-T regardless of A-[CO₂] (Figure 1A, B, E, and F) or E-[CO₂] (Fig. C, D, G, and H), suggesting that the oxalate contents of leaves were not affected by a 2°C increase in soil and water temperature.

To investigate which metabolite or pathway affects oxalate accumulation, correlation analysis between oxalate and its related metabolite content was performed using the metabolite data set described in the previous study (Noguchi et al., 2018). Higher positive correlation between oxalate and the organic acids, involved in the TCA cycle (e.g. citrate, isocitrate, 2-oxoglutarate, and succinate) and shikimate, were shown in all Koshihikari and Takanari leaves (Figure 2), while negative correlation was observed between oxalate and amino acids (such as glutamate and aspartate). This suggests that the flows of carbon hydroxylate used for oxalate synthesis were upregulated in Koshihikari, although amino acids were more synthesised in Takanari. For both ascorbate and glycine, absolute values of correlation coefficient were small if the cultivars were lumped together, but a negative correlation between ascorbate and oxalate was observed in Koshihikari ($R = -0.545$, $P < 0.01$), but not in Takanari ($R = -0.144$), indicating that ascorbate pathway may affect oxalate accumulation in Koshihikari. The positive correlation between glycine and oxalate was observed not in Takanari ($R = 0.043$) but in Koshihikari ($R = 0.530$, $P < 0.01$). The contents of these metabolites, related to photorespiration and oxalate synthesis, were altered by high [CO₂] in Koshihikari. The alteration of these metabolites by high [CO₂] may contribute to slight reduction of oxalate content in Koshihikari.

In conclusion, the varietal differences in the leaf oxalate contents between Koshihikari and Takanari were maintained regardless of the environmental factors such as E-[CO₂] and/or E-T conditions. Under high [CO₂] conditions, a slight decrease in oxalate contents was observed in Koshihikari leaves, presumably owing to the inhibition of photorespiration. However, the results suggest that the glycolate and/or ascorbate pathway may not be responsible for the observed varietal differences in the oxalate content. Future experiments involving molecular genetic analysis combined

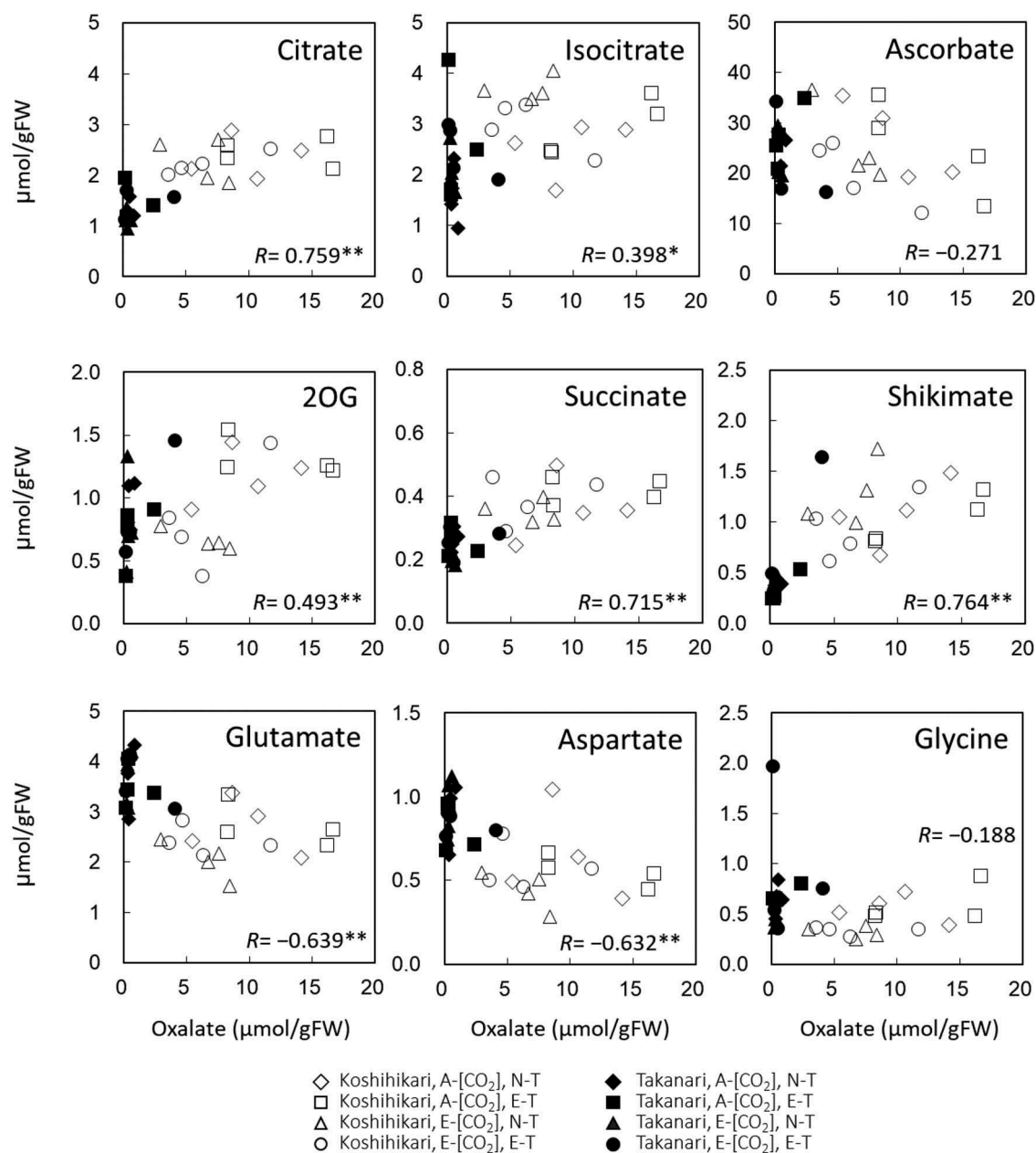


Figure 2. Correlation analysis between oxalate and other metabolites using the metabolite data sets (except for oxalate) in Noguchi et al. (2018). A-[CO₂]; ambient [CO₂], E-[CO₂]; elevated [CO₂], N-T; normal temperature, E-T; elevated soil/water temperature, *, $P < 0.05$, **, $P < 0.01$.

with metabolomic and transcriptomic analyses may clarify the oxalate dynamics in the leaves of Koshihikari and Takanari cultivars.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The present study was supported by MEXT Kakenhi Grant Numbers JP16K20953 and JP18K14386, Japan, CREST, the

Science and Technology Agency (JST), Japan, and the Ministry of Agriculture, Forestry and Fisheries, Japan through the research project 'Development of Technologies for Mitigation and Adaptation to Climate Change in Agriculture, Forestry and Fisheries'. The SAS was run on the supercomputer of AFFRIT, MAFF, Japan.

ORCID

Takeshi Tokida <http://orcid.org/0000-0001-7245-2952>

Toshihiro Hasegawa <http://orcid.org/0000-0001-8501-5612>

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