Running title: Carbon cycle mediated by C1-microbes and plants

Interaction between C1-microorganisms and plants: contribution to the global carbon cycle and microbial survival strategies in the phyllosphere

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Abstract

C1-microorganisms that can utilize C1-compounds, such as methane and methanol, are ubiquitous in nature, and contribute to drive the global carbon cycle between two major greenhouse gases, CO_2 and methane. Plants emit C1-compounds from their leaves and provide habitats for C1-microorganisms. Among C1-microorganisms, *Methylobacterium* spp., representative of methanol-utilizing methylotrophic bacteria, predominantly colonize the phyllosphere and are known to promote the plant growth. This review summarizes the interactions between C1-mircroorganisms and plants that affect not only the fixation of C1-compounds produced by plants but also CO_2 fixation by plants. We also describe our recent understanding of the survival strategy of C1-microorganisms in the phyllosphere and the application of *Methylobacterium* spp. to improve rice crop yield.

Keywords

methanol, methylotorph, phyllosphere, *Methylobacterium*, plant growth promotion

1. Introduction

Among compounds that have no carbon-carbon bond, the most oxidized compound CO_2 and the most reduced compound methane are two major greenhouse gases. Methane is the second most abundant greenhouse gas after CO_2, and contributes approximately 20% to global warming induced by long-lived greenhouse gases since pre-industrial times (Kirschke *et al.* 2013). The global carbon cycling between these two gases is called methane cycle (Fig. 1). According to several reports regarding the global methane budget, annual emission and sink of methane are estimated to be 560 Tg and 550 Tg, respectively (Kirschke *et al*. 2013; Saunois *et al.* 2016). Methane is produced by methanogenic archaea in anaerobic environments including those of anthropogenic origins, such as paddy fields. Most of the atmospheric methane (more than 80%) is oxidized by the hydroxy radical in the troposphere (Guenther 2002). In the methane cycle, biological oxidation of methane to CO_2 is conducted by a group of microorganisms called C1-microorganisms (methylotrophs) that can utilize reduced C1-compounds, including methane and methanol, as the sole source of carbon and energy. Primary oxidation of methane is performed not only by aerobic methanotrophic bacteria that are methane-utilizing methylotrophs, but also by anaerobic methanotrophic (ANME) archaea (Knief 2019). Methanotrophic bacteria oxidize methane generated in anoxic environments before it reaches the atmosphere as well as the atmospheric methane (Aronson, Allison and Helliker 2013).

C1-microorganisms inhabit various natural environments. Recently much attention has been paid to the above-ground part of plants called phyllosphere as major habitats for C1-microoganisms, since it has been reported that huge amount of methane and methanol are emitted from living plants (Fall and Benson 1996; Keppler *et al.* 2006; Nemecek-Marshall *et al.* 1995). Among microorganisms living in the phyllosphere, methanol-utilizing methylotrophic bacteria, *Methylobacterium* spp., also known as pink-pigmented facultative methylotrophs (PPFMs), are dominant colonizers and some of

them are known to promote the plant growth (Dourado *et al.* 2015; Knief *et al.* 2012; Knief *et al.* 2010; Kumar *et al.* 2016). While plant-rhizobia and plant-mycorrhizae interactions in the root environments (rhizosphere) have been investigated for a long time, interactions between PPFMs and plants in the phyllosphere have come to be investigated in the last two decades. Furthermore, recently PPFMs are considered to contribute not only to the oxidation process from methane to CO_2 in the global carbon cycle but also to CO_2 fixation by plants (Fig. 1).

In this minireview, we summarize the carbon cycle mediated by C1-microorganisms and plants and describe the current understanding of survival strategies of PPFMs in the phyllosphere. Finally, we also discuss the application of PPFMs to improve rice crop yield.

2. Emission of C1-compounds from plants and plant colonization of C1-microorganisms

Methanol that originates from methylesters in the plant cell wall constituent pectin is emitted from plant leaves and its annual emission is estimated to be 100 Tg (Fall and Benson 1996; Galbally and Kirstine 2002; Henrot *et al.* 2017). The concentration of methanol emitted from plants to the air phase has been reported to fluctuate depending on the opening and closing of stomata (Nemecek-Marshall *et al*. 1995). But we reported that the concentration of methanol available for microorganisms on the surface of *Arabidopsis thaliana* leaves oscillates during the daily light–dark cycle (Kawaguchi *et al.* 2011). By using a cell-based methanol sensor of the methylotrophic yeast *Candida boidinii* in which the fluorescent protein is expressed under the methanol-induced gene promoter, we showed that the methanol concentration in the phyllosphere was higher in the dark period and lower in the light period, which was opposite to that of atmospheric methanol. We think that methanol, which is accumulated in the spongy parenchyma of the leaf during stomatal closing in the dark period, diffuses to the surface of the leaf.

The global leaf area is estimated to be twice as large as the surface of the earth and provides habitats for bacterial populations of 10^{26}–10^{27} cells, as well as for lower numbers of archaea and fungi (Lindow and Brandl 2003). On such huge phyllospheric environment, methanol-utilizing *Methylobacterium* is a major genus among phyllospheric bacteria; for example, this genus has been shown to be the most dominant in both dicots and monocots, such as soybean, clover, and rice (Delmotte *et al.* 2009; Knief *et al*. 2012).

Methane emission from plants was firstly reported in 2006 (Keppler *et al*. 2006). In addition to the methane emitted via aerenchyma from soil environments, some plants aerobically produce methane, which is assumed to be formed during the synthesis of pectin methyl ester groups along with photosynthesis (Aulakh *et al.* 2000; Bruhn *et al.* 2012). Annual emission of methane from plants is estimated to be 10-69 Tg (Butenhoff and Khalil 2007; Kirschbaum *et al.* 2006; Parsons *et al.* 2006). According to some previous reports, metagenomic and metaproteomic analyses did not detect methanotrophs on leaves of soybean, clover, *A. thaliana,* and so on (Delmotte *et al*. 2009; Yang *et al.* 2001), but other studies detected small populations on leaves of rice, soybean*,* and so on (Finkel *et al.* 2011; Ikeda *et al*. 2011; Knief *et al*. 2012). Recently we demonstrated that methanotrophs could be cultivated from 12% of the phyllosphere samples (Iguchi *et al.* 2012). Furthermore, we found that both submerged and floating aquatic plants serve as a niche for methanotrophs and that these hydrophytes associated with methanotrophs have higher methane oxidation activity than emergent parts of plants (Iguchi *et al.* 2019; Yoshida *et al.* 2014).

3. Distribution of PPFMs in the phyllosphere and species level specificity between PPFMs and plants

Although a number of PPFM strains have been isolated from plant-related materials, the species-level distribution of PPFMs in the phyllosphere and the species-level

114 specificity between PPFMs and plants have not been well-understood until recently. We
115 investigated the distribution of PPFMs on the leaves of various vegetables, and revealed
116 that the number of PPFMs on the leaves differed among the plants although they were
117 grown at the same farm (ca. 100 m^2) (Mizuno *et al.* 2012). Thus, the plant species
118 affects the population size of PPFMs on leaves. Furthermore, we found that PPFMs
119 were highly abundant on leaves of green perilla (*Perilla frutescens viridis* (Makino)
120 Makino) and red perilla (*Perilla frutescens crispa* (Thunb.) Makino). The PPFMs
121 isolated from red perilla seeds harvested in different years and those isolated from red
122 perilla leaves planted at four geographically different places in Japan had 16S rRNA
123 sequences identical to that of the representative strain *Methylobacterium* sp. OR01
124 isolated from red perilla seeds (Mizuno *et al.* 2013). These results indicate the
125 geographically independent species-level specific PPFM-perilla plant associations. We
126 also confirmed the direct transmission of *Methylobacterium* sp. OR01 from red perilla
127 seeds to their leaves by using the antibiotics-resistant strain OR01 (Mizuno *et al*. 2013).
128
129 **4. Nutrient sources for PPFMs in the phyllosphere**
130 In the phyllosphere, PPFMs utilize compounds supplied by plants as their nutrient
131 sources for growth and survival (Fig. 2). The ability of PPFMs to utilize methanol is
132 thought to be one of the reasons why these are the dominant bacteria colonizing the
133 phyllosphere. In previous studies using the representative model strain
134 *Methylobacterium extorquens* AM1, (which has recently been re-classified as
135 *Methylorubrum extorquens* (Green and Ardley 2018)), mutant strains lacking *mxaF* or
136 *xoxF*, which encode the large subunit of Ca^{2+}-dependent methanol dehydrogenase
137 (MDH) or a lanthanides-dependent MDH, respectively, were shown to be less
138 competitive than the wild-type strain for colonizing plant leaves (Schmidt *et al.* 2010;
139 Sy *et al.* 2005). These results suggest that the ability to utilize methanol as a carbon
140 source is advantageous for PPFMs for growth and survival in the phyllosphere. On the

141 other hand, these mutant strains were still able to colonize plant leaves, indicating that
142 PPFMs utilize other carbon sources besides methanol in the phyllosphere. Indeed, the
143 presence of sugar compounds including glucose on the leaf surface has been reported
144 (Mercier and Lindow 2000).
145 Some trace cofactors such as vitamins have also been reported to be present on the
146 leaf surface (Gargallo-Garriga *et al.* 2016), and can be utilized by PPFMs in the
147 phyllosphere (Rodionov *et al.* 2009). Recently, a number of PPFMs isolated from living
148 plant samples, including *Methylobacterium* sp. OR01, were found to require B vitamins
149 for their growth on a minimal medium, and most B vitamin-auxotrophic PPFMs
150 required pantothenate (vitamin B5) (Yoshida *et al.* 2019). Further analysis revealed that
151 *Methylobacterium* sp. OR01 could not synthesize β-alanine, which is one of the
152 precursors of pantothenate biosynthesis. β-Alanine and its biosynthetic precursors,
153 spermine, spermidine, 5,6-dihydrouracil, *N*-carbamoyl-β-alanine, and 3-
154 hydroxypropanoate, restored the growth of *Methylobacterium* sp. OR01 in minimal
155 medium. This strain could colonize leaves of *A. thaliana* cultivated on a plant medium
156 without pantothenate or its precursors, and furthermore, pantothenate, β-alanine and
157 several precursor compounds were detected in the slight wash solution of *A. thaliana*
158 leaves. These results suggest that pantothenate-auxotrophic PPFMs colonize the
159 phyllosphere by utilizing not only pantothenate, but also β-alanine and some other
160 precursors produced by the host plants.
161 When the plant colonization ability between the pantothenate auxotrophic
162 *Methylobacterium* sp. OR01 and *M. extorquens* AM1, which is prototrophic for not only
163 pantothenate but also other B vitamins, were compared, *Methylobacterium* sp. OR01
164 was found to dominate over the non-auxotrophic strain AM1 on *A. thaliana* leaves
165 (Yoshida *et al.* 2019). The auxotrophic *Methylobacterium* sp. OR01 can save the energy
166 costs of the biosynthesis of pantothenate or β-alanine. Thus, the fitness advantage of the
167 auxotrophic strain increased more than that of the prototrophic strain. This hypothesis is

supported by the recent report that half of the bacterial strains isolated from *A. thaliana* leaves had auxotrophic requirements for biotin, niacin, pantothenate, and/or thiamine (Ryback, Bortfeld-Miller and Vorholt 2022).

5. Survival strategies of PPFMs to adapt to phyllosphere environments

The phyllosphere is thought to be a harsh environment and PPFMs in the phyllosphere are exposed to various kinds of environmental factors, such as diurnal temperature change, UV radiation, drought, osmotic pressure, reactive oxygen species (ROS), and low nutrients. As described above, methanol concentration on the leaf surface oscillates diurnally. Therefore, PPFMs must have some survival strategies to adapt to these diurnally changing environmental factors (Fig. 2). One such strategy is to regulate stress-response genes. It was reported that PhyR, which is a general stress response regulator, is involved in plant colonization of *M. extorquens* AM1 and other α-proteobacteria (Gourion, Francez-Charlot and Vorholt 2008; Gourion, Rossignol and Vorholt 2006; Gourion *et al.* 2009). PhyR was first identified as a more abundantly produced protein by the proteome analysis of *M. extorquens* AM1 in the phyllosphere than in the rhizosphere. The *phyR* mutant strain was shown to be deficient in its plant colonization ability and was also shown to increase sensitivity to various stresses, such as heat, UV light, osmolarity, and ROS (Gourion, Francez-Charlot and Vorholt 2008; Gourion, Rossignol and Vorholt 2006). Thus, PhyR appears to be essential for plant colonization and the general stress response system regulated by PhyR might contribute to enhanced fitness in phyllosphere environments. We also revealed that PhyR was involved in resistance to heat shock and UV light in a methanotroph, *Methylosinus* sp. B4S isolated from a plant leaf (Iguchi *et al.* 2013).

Not only nutrients available for PPFMs, but also environmental conditions such as temperature and sun light diurnally change in the phyllosphere. We have investigated the physiological role of *M. extorquens* AM1 KaiC proteins, which are homologues of

195 the component of circadian clock generator in cyanobacteria (Iguchi *et al.* 2018). KaiC
196 proteins in cyanobacteria have both autokinase and autophosphatase activities and the
197 phosphorylation level of KaiC exhibits an environment-independent oscillation with a
198 24 h period (Johnson, Mori and Xu 2008; Johnson *et al.* 2017). The Kai protein
199 complex (KaiA, KaiB, and KaiC) regulates global gene expression through downstream
200 regulators such as LabA. *M. extorquens* AM1 has two KaiC homologues, KaiC1 and
201 KaiC2, in which serine residues corresponding to the phosphorylation sites of the
202 cyanobacterial KaiC are conserved. We tested competitive colonization between the
203 wild-type and gene-disrupted strains on *A. thaliana* and revealed that KaiC2 and LabA
204 are necessary for optimal colonization of *M. extorquens* AM1 in the phyllosphere
205 (Iguchi *et al*. 2018). In addition, the phosphorylation-defective mutant KaiC2m was
206 unable to restore the colonization ability of the Δk*aiC2* strain, indicating that
207 phosphoregulation of KaiC2 is important for colonization on plants.
208 *M. extorquens* AM1 exhibits temperature-dependent UV resistance (TDR). The
209 survival ratio of the wild-type strain after UV treatment has been shown to increase with
210 increasing growth temperatures (24–32 °C) (Iguchi *et al*. 2018). Further analyses
211 revealed that the TDR phenotype was positively regulated by KaiC1 and negatively
212 regulated by KaiC2. Based on the analyses of KaiC1 and KaiC2 protein levels and their
213 phosphorylation status at different temperatures, we concluded that the amount of KaiC
214 proteins and the phosphorylation state of KaiC2 control the UV resistance pathway in
215 an integrated manner according to the growth temperature, thus allowing cells to adapt
216 to changing environmental conditions.
217
218 **6. Positive interaction between plant and PPFMs: Improvement of rice crop yield**
219 **in paddy fields**
220 Some PPFMs are known to enhance seedling growth and total biomass of various
221 plants. Plant growth promotion by PPFMs is thought to be achieved by the following

222 characteristics of PPFMs (Fig. 2) (Dourado *et al*. 2015; Yurimoto, Shiraishi and Sakai
223 2021); i) they produce phytohormones, such as auxins and cytokinins, and the inhibitor
224 of ethylene biosynthesis (i.e., 1-aminocyclopropane-1-carboxylate (ACC) deaminase)
225 (Ortiz-Castro *et al*. 2009). ii) they induce systemic plant resistance against pathogens
226 and diseases (Madhaiyan *et al*. 2004). iii) they also facilitate improvements in uptake of
227 plant nutrients with their involvement in functions such as siderophore production,
228 phosphate solubilization, and N_2 fixation (Kumar *et al*. 2019). There have been
229 scattered reports on improvement on yield by treatment with PPFMs (via seed
230 inoculation or foliar spraying) under laboratory conditions or pot-scale cultivation,
231 particularly for vegetables (Abanda-Nkpwatt *et al.* 2006; Ryu *et al*. 2006; Madhaiyan *et*
232 *al*. 2006; Meena e*t al*. 2012). However, improvement of crop yields by inoculation of
233 PPFMs at the field level has not been well investigated. Recently, foliar spraying of
234 PPFMs was found to improve rice crop yields in a commercial paddy field (Yurimoto *et*
235 *al.* 2021). The crop yield of the sake-brewing rice cultivar Hakutsurunishiki was
236 improved by foliar spraying of PPFMs in a commercial paddy field for over a five-year
237 period. Interestingly, foliar spraying of not only living cells but also killed cells or a cell
238 wall polysaccharide fraction gave positive effects on the rice crop yield (Fig. 3a). After
239 optimization of the timing of PPFM inoculation, a one-time foliar spray of killed PPFM
240 cells after the heading date was found to be effective in increasing the rate of ripening
241 (Fig. 3b) and crop yield (16% increase in the unit yield, Fig. 3c). We also observed the
242 greenization of rice seedling leaves by PPFM spraying, possibly due to the increase in
243 plant chlorophyll content leading to an enhancement in photosynthetic activity. The
244 mechanism of how PPFMs affect the rice crop yield after the heading date is still
245 unclear; however, we speculate that a direct interaction of PPFM cell wall components
246 with the plant might stimulate the plant cells to enhance photosynthetic activity during
247 the translocation stage of rice growth (Yurimoto *et al*. 2021).
248

7. Conclusion and future perspectives

In this review, we described our recent understanding of the interaction between C1-microorganisms and plants, particularly on the survival strategies of PPFMs in the phyllosphere and improvement of rice crop yield by them. Positive interactions between PPFMs and plants affect the global carbon cycle both through fixation of C1-compounds produced by plants and CO_2 fixation by plant photosynthesis (Fig. 1). We still need to better understand the basis of symbiotic interactions between methylotrophs and plants at the molecular level.

The practical use of the positive interactions between PPFMs and plants will lead to development of new technologies both in agriculture and in environmental sustainability. Since PPFM cells can be cultivated at high-cell density with methanol as a carbon source (Schrader *et al.* 2009), which can be derived from methane or renewable biomass, it is easy to prepare large amounts of cells for use in the field. Application of PPFMs to agriculture has the potential to reduce CO_2 emission. Thus, the prospects of C1-microorganisms playing extremely important roles in the global carbon cycle are high.

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Author's Contribution

HY and YS designed and wrote the manuscript.

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Figure legends

Figure 1. The global carbon cycle mediated by C1-miroorganisms and plants. Methane is generated from CO_2 by methanogens and C1-microorganisms including methanotrophs and methanol-utilizing methylotrophs oxidize methane and other C1 compounds to CO_2. This cycle is known as methane cycle. Recently, C1-microorganisms in the phyllosphere were found to utilize methane and methanol produced by plants. Positive interactions between PPFMs and plants enhance CO_2 fixation and increase plant biomass (yield increase).

Figure 2. Interactions between PPFMs and plants in the phyllosphere. PPFMs utilize nutrients such as methanol as a carbon source and other cofactors such as vitamins. PPFMs provide benefit to plants by producing plant hormones, enhancing nutrient uptake of plants, and inducing resistance to pathogens. PPFMs have various cellular functions for adapting to diurnally changing environmental factors in the phyllosphere.

Figure 3. Summary of the effects of PPFM treatment on rice crop yields (cultivar Hakutsurunishiki). Graphs were replotted from the previously reported data (Yurimoto *et al*. 2021). (a) The weight of brown rice in a commercial paddy field in 2017 following the indicated treatments. (b and c) The rate of ripening (b) and the unit yield (c) in 2018 after the indicated treatments.

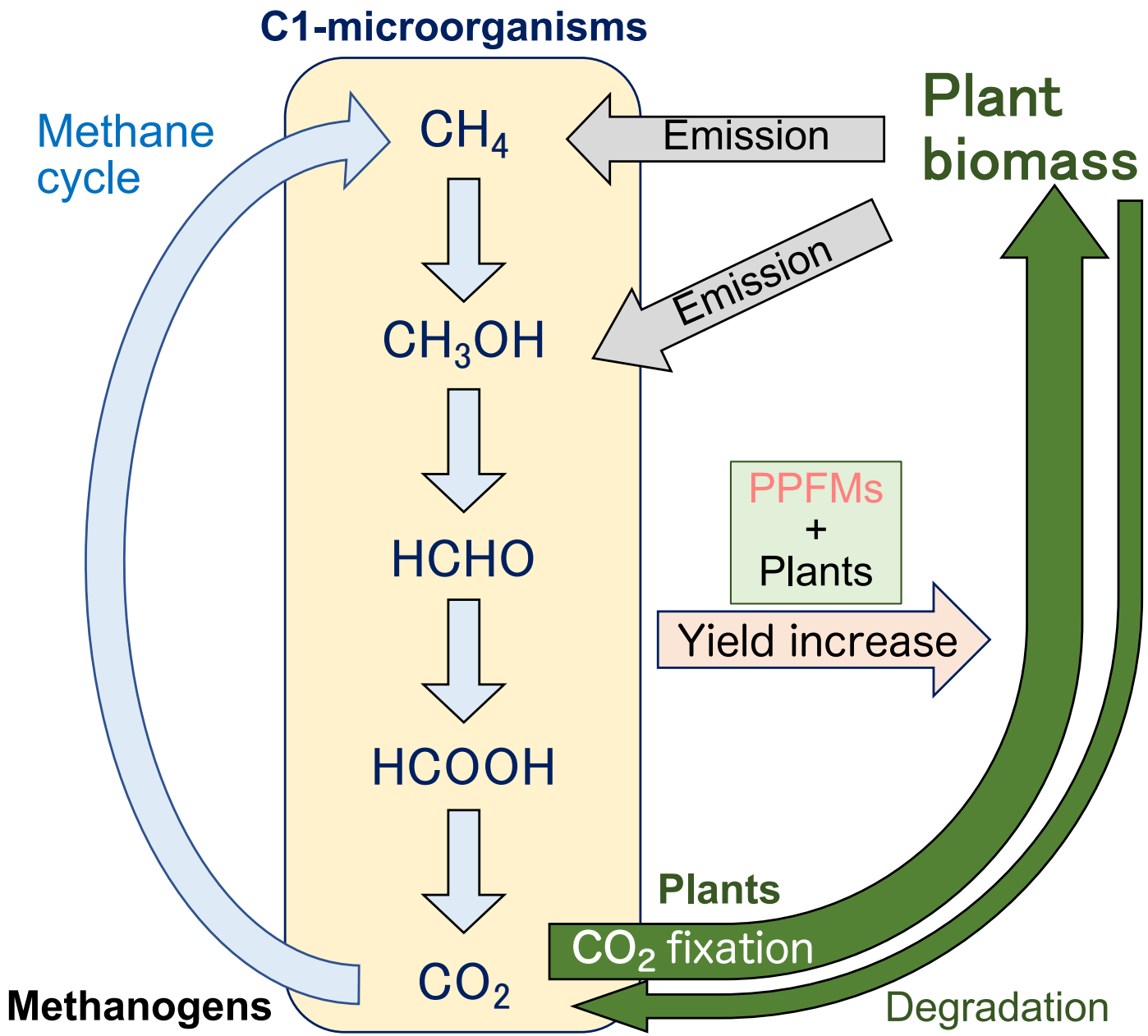
C1-microorganisms
Methane cycle
CH_4
Emission
Plant biomass
Emission
CH_3OH
HCHO
PPFMs + Plants
Yield increase
HCOOH
Plants
CO_2 fixation
CO_2
Methanogens
Degradation

Fig. 1

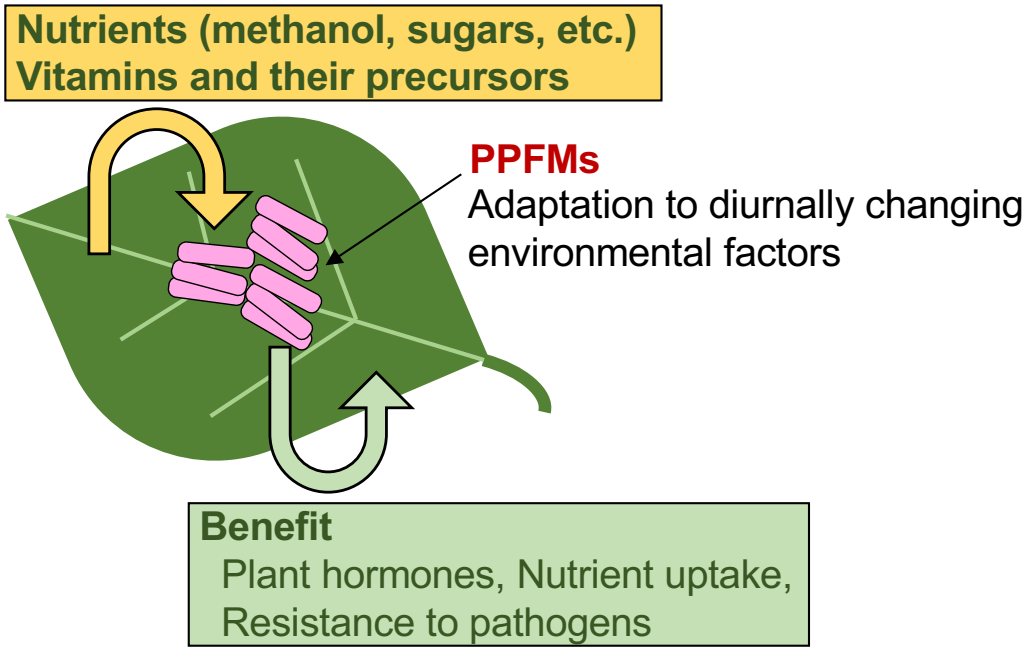
Nutrients (methanol, sugars, etc.)
Vitamins and their precursors
PPFMs
Adaptation to diurnally changing environmental factors
Benefit
Plant hormones, Nutrient uptake,
Resistance to pathogens

Fig. 2

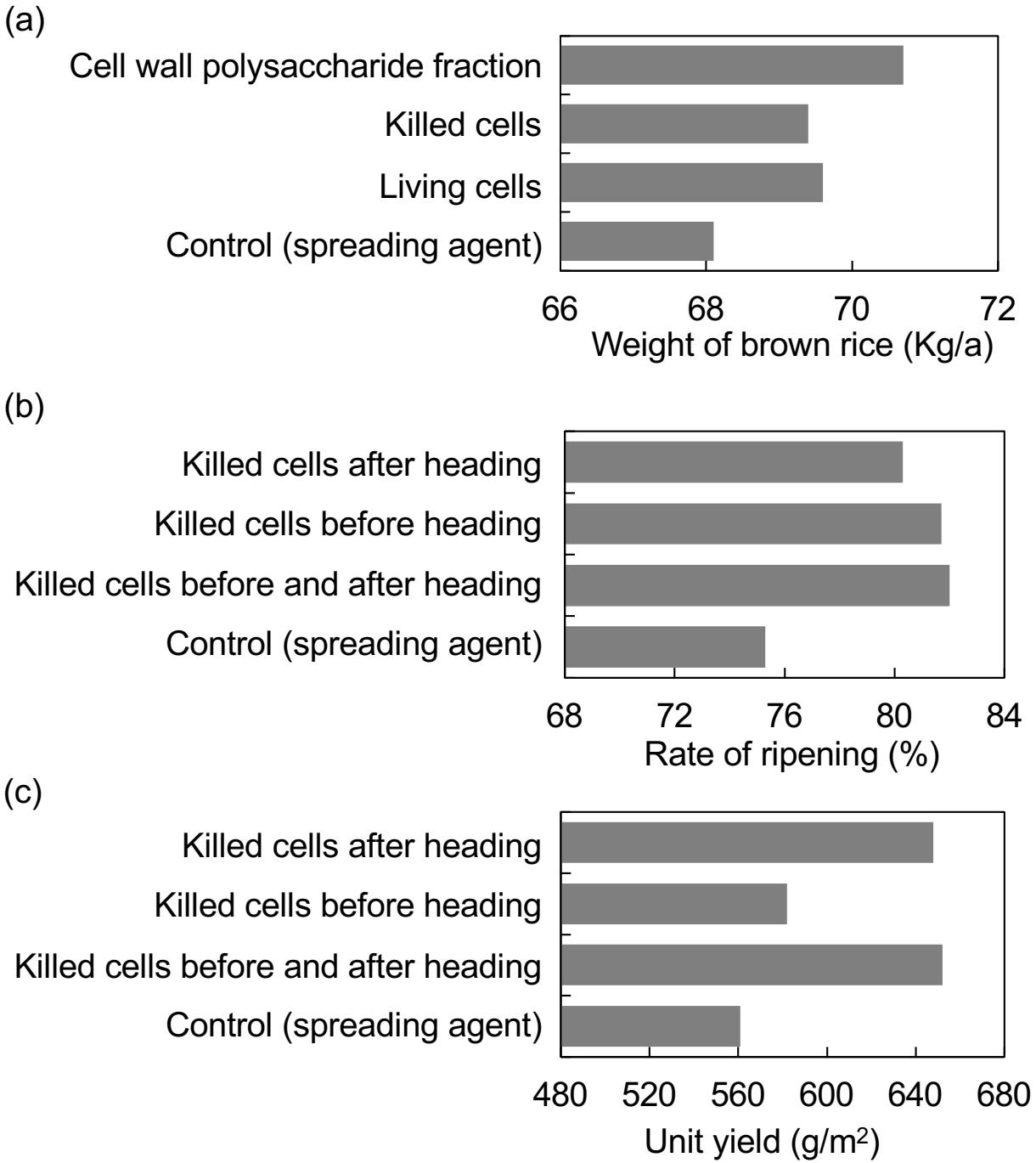
(a)
Cell wall polysaccharide fraction
Killed cells
Living cells
Control (spreading agent)
66
68
70
72
Weight of brown rice (Kg/a)
(b)
Killed cells after heading
Killed cells before heading
Killed cells before and after heading
Control (spreading agent)
68
72
76
80
84
Rate of ripening (%)
(c)
Killed cells after heading
Killed cells before heading
Killed cells before and after heading
Control (spreading agent)
480
520
560
600
640
680
Unit yield (g/m2)

Fig. 3