**Anonymous Referee #2**

Received and published: 23 May 2018

This is a problem-based research and the experiment is well designed. The authors found that an ethylene biosynthesis inhibitor (AVG) increased the methane oxidation rates. The manuscript is short but well written. It should be changed to a short communication. I just have few minor comments and questions.

R: Thanks for your positive comments. The type of the manuscript has been changed into 'technical note', a kind of short communication for this journal.

1.Why did not determine the methane oxidation rates in situ? The incubation only shows the potential rates. 2.The authors sampled the soils in late June. It will be better If they can sample for several times from April to June.

R: Yes, you are right. It is very unfortunate that I got some personal healthy issue while doing this experiment, so we only collected one sampling time of soil samples before crop harvest and did incubation to measure soil methanotrophic activities. However, our results provide good evidence for our previous hypothesis that drought-induced *in planta* ethylene production reduces soil $CH_4$ oxidation rates (Zhou et al., 2013). In summary, we do acknowledge that *in situ* $CH_4$ efflux data and/or more sampling times would have been better, but unforeseen circumstances did not allow it.

3. L162: What is "produce $CH_4$ oxidation rates"?

R: It has been revised. Line 190

# Technical Note

# Manipulating interactions between plant stress responses and soil methane oxidation rates

Xiaoqi Zhou[1,2,3], Chengyuan Xu[2,4], Shahla H Bai[2,5], Zhihong Xu[2], Simeon J Smaill[6*], Peter W Clinton[6], Chengrong Chen[2,3]

[1]Tiantong National Station for Forest Ecosystem Research, Center for Global Change and Ecological Forecasting, Shanghai Key Lab for Urban Ecological Processes and Eco-restoration, Institute of Eco-Chongming, School of Ecological and Environmental Sciences, East China Normal University, Shanghai 200241, China

[2] Environmental Futures Research Institute, Griffith University, Nathan, Brisbane, 4111, Australia

[3] Griffith School of Environment, Griffith University, Nathan, Brisbane, 4111, Australia

[4] School of Medical and Applied Sciences, Central Queensland University, Bundaberg, QLD, 4760, Australia

[5] Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Maroochydore, DC Qld 4558, Australia

[6] Scion, PO Box 29237, Riccarton, Christchurch 8440, New Zealand

[*] Corresponding author: Dr. Simeon J Smaill

simeon.smaill@scionresearch.com

**Abstract**

It has recently been hypothesised that ethylene, released into soil by stressed plants, reduces the oxidation of methane by methanotroph. To test this, a field trial was established in which maize plants were grown with and without soil moisture stress, and the effects of addition aminoethoxyvinylglycine (AVG; an ethylene biosynthesis inhibitor), and biochar (increases soil water holding capacity and reduces plant stress) were determined following the static incubation of soil samples. AVG increased methane oxidation rates by 50% (P=0.039), but only in the absence of irrigation. No other treatment effects were observed. This result provides evidence for a positive feedback system between plant stress, ethylene production, and impacts on methanotrophic activity.

**Keywords** methane oxidation; plant stress; ethylene inhibition; methanotroph; positive feedback

## 1 Introduction

The atmospheric concentration of methane ($CH_4$) has almost tripled over the past 150 years, making a substantial contribution to climate change (Forster et al., 2007). Aerobic soils provide an important habitat for methanotrophic bacteria, and provide the only significant biological sink for atmospheric $CH_4$ (20-45 Tg $CH_4$ $yr^{-1}$) (Forster et al., 2007). However, $CH_4$ uptake by these soil ecosystems can be impacted by environmental stress (Kolb, 2009). A common plant physiological response to ecological stress, such as drought, is the production of ethylene (Morgan and Drew, 1997). In soils, however, ethylene may be inhibitory to methanotrophic activity (Jäckel et al., 2004; Pierek et al., 2006; Zhou et al., 2013), and thereby reduce $CH_4$ oxidation. This potential interaction needs to be understood, as it may constitute an important positive feedback loop between climate disruption, soil ecosystem disturbance, and reduced $CH_4$ removal from the atmosphere (Bousquet et al., 2006; Zhou et al., 2013).

To test our previous hypothesis that drought-induced *in planta* ethylene production reduces soil $CH_4$ oxidation rates (Zhou et al., 2013), we manipulated plant stress responses by adding the ethylene biosynthesis inhibitor aminoethoxyvinylglycine ([S]-trans-2-amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride; hereafter AVG) (Boller et al., 1979). In addition, the study tested the hypothesis that addition of biochar to soils may result in increased water holding capacity, reducing drought stress and thereby acting as a potential tool to maintain $CH_4$ oxidation (Karhu et al., 2011). This is illustrated conceptually in Fig. 1, in which the application of irrigation (IR) and biochar (BC) are able to maintain rates of $CH_4$ oxidation by reducing moisture stress and therefore ethylene production, whereas AVG prevents the production of ethylene after the plant experiences stress.

## 2 Material and methods

### 2.1 Study site

The study site was located in the Bjelke-Petersen *Research Station* at Kingaroy (26.53° S, 151.83° E) in the South Burnett Region of Queensland, Australia. Precipitation averages 789 mm per annum with erratic summer droughts frequent in the region. Soil at the field trial site soil is an acidic red ferrosol (pH 5.5) with high cation

89 exchange capacity (Isbell, 1993). The site has a long history of cultivation, supporting
90 peanut and maize rotations with winter fallows.

91 **2.2 Experiment design and management**

92 A full factorial, split plot design field trial was established as follows: two IR
93 treatments (IR and no IR) × two BC treatments (BC at 9.2 t $ha^{-1}$ and no BC) × two
94 ethylene suppression treatments (AVG and no AVG). Each treatment had five replicates,
95 producing a total of 40 plots. Due to practical concerns regarding application and
96 maintenance, the IR treatments were established in two discrete areas that were spanned
97 by five blocks. A schematic of the trial site is given in the supplementary information
98 (Fig. S1).

99 The BC treatment was established through application of peanut shell BC to the
100 surface of the planting zone (~450 mm wide strip each row) in early 2013. The BC was
101 incorporated into the soil with a rotary hoe to a depth of 200 mm. The chemical
102 properties of the peanut shell BC are provided in the supplementary information (Table
103 S1).

104 The site was machine planted with maize cultivar Pioneer 32p55 (Dupont Pioneer
105 Australia) at a density of approximately 4 plants $m^{-2}$ in late January, 2014. Compound
106 fertiliser ($N:P_2O_5:K_2O$ 11.9:14.1:9.9) at 180 kg $ha^{-1}$, and urea at 100 kg $ha^{-1}$, were
107 applied at sowing. Trickle tapes, installed into plots receiving IR, were used to distribute
108 water equivalent to ~50 mm of rainfall whenever there was a continuous dry spell for
109 two weeks throughout the growing season (late January to late June).

110 To reduce the *in planta* production of ethylene, the commercial plant growth
111 regulator ReTain (containing 15% AVG, Valent Bioscience Cooperation, Walnut Creek,
112 CA, USA) was sprayed onto the crop four times from mid-April to mid-June (the peak
113 maize growth window) at intervals of three weeks. During each event, the treated rows
114 of maize received approximately 750 ml of ReTain solution (prepared at the label rate
115 of 1 g ReTain $l^{-1}$ water) directly to the surface of the plants.

116 **2.3 Sample collection and analysis**

117 In late June 2014, six soil cores from 0 - 100 mm depth were collected from the maize
118 rooting zone of each plot using a 30 mm diameter soil auger. All samples were collected

from the two middle rows of maize in each plot, and the six soil cores from within each plot bulked to a single plot sample. After sieving to 2 mm, a 50 g (fresh mass) subsample of each sample was set aside for $CH_4$ oxidation rate measurements and the remaining material dried at 105°C for 48 hours to determine soil moisture content.

Soil $CH_4$ oxidation rates were determined using the laboratory incubation. Briefly, about 20 g soil subsamples were incubated in 1-litre glass jars at ambient atmospheric $CH_4$ concentration (assumed to be 1.9 ppm) for one week in the dark at 25°C. Headspace gas samples (approximately 30 ml) were collected through a rubber septum in the jar lid at the beginning and the end of the incubation, and concentrations of $CH_4$ was determined using GC-FID (GC-2010 Plus, Shimadzu, Japan). The $CH_4$ oxidation rates in each jar were calculated from differences in the headspace $CH_4$ concentration over the incubation time (Zhou et al., 2008), and adjusted to soil dry weight. Standards were measured once every 10 samples; the coefficient of variation in $CH_4$ oxidation rate was less than 5% and control jars had ambient $CH_4$ concentrations.

### 2.4 Statistical analysis

Statistical analysis was carried out in R-3.2.3 (Zhou et al., 2017) using a multi-factor ANOVA model incorporating an error structure accounting for the split-plot design associated with the non-random assignment of the IR treatment. The multi-comparison analysis methods provided in the "easyanova 4.0" R package was used to test for treatment interactions.

## 3 Results

Over the course of the field trial, five dry spells occurred. Irrigation to the IR-plots resulted in delivery of 250 mm more water to this treatment than the controls. This resulted in significantly increased soil moisture ($P<0.001$) in IR soils (18.9%) compared with the non-irrigated soils (15.4%) at sampling time. Neither the AVG or BC treatments had any effect ($P>0.05$) on soil moisture.

No significant main effects were observed, but a significant interaction between irrigation and AVG application was detected (Table 1). Exploration of this interaction with multi-comparison analysis determined that $CH_4$ oxidation rates were increased by 50% following AVG application ($P=0.039$), but only in the absence of IR (Fig. 2). The

addition of biochar had no effect on $CH_4$ oxidation rates either as a main or interactive effect.

## 4 Discussion

The increase in $CH_4$ oxidation with the AVG treatment either alone or in combination with the BC treatment aligns with past studies assessing the effect of increased ethylene concentrations on soil $CH_4$ oxidation rates (Jäckel et al., 2004; Xu et al., 2008). This response also supports the hypothesis that *in planta* ethylene production in response to stress decreases the capacity of soil to support methanotrophic activity (Zhou et al., 2013).

The lack of effect of BC on $CH_4$ oxidation is at odds with the results of previous work (e.g. Karhu et al., 2011; Kim et al., 2017). However, BC added in this study had no influence on soil moisture content, and this is proposed to be a key mechanism for BC to support $CH_4$ oxidation in drought conditions (Karhu et al., 2011). Another reason for this might be related to the properties of the biochar (C:N ratio of 51.84, 9.2 t $ha^{-1}$) used in this study when compared with agricultural soils in Finland (C:N ratio of 101.07, 9 t $ha^{-1}$) (e.g. Karhu et al., 2011) and in East Asia (C:N ratio of 79.65, 2 t $ha^{-1}$) (e.g. Kim et al., 2017). The lower C:N ratio of the biochar used in this study can incorporate more N fertilizer into the soils, which could reduce soil $CH_4$ uptake as N fertilizer can inhibit methanotrophic activities (see Kolb, 2009). Overall, the reason why BC addition did not result in increased soil moisture in this case is unclear. Further studies is needed to investigate the effects of biochar application on the factors influencing soil $CH_4$ oxidation.

The significant interaction between the AVG and IR treatments is more difficult to reconcile. The IR treatment was intended to significantly increase soil moisture content compared to the no IR treatment, reducing water stress and likely *in planta* ethylene production. It was noted that increased soil moisture content can directly influence methanotrophic activity, as water-driven increases in microbial activity can enhance methanotroph, whereas water content that exceed field capacity can rapidly decrease $CH_4$ oxidation rates by reducing gas mobility through soil pores (Le Mer and Roger., 2001). Given the initial soil water content and scale of the increase with the IR treatment,

179 direct stimulation of $CH_4$ oxidation was considered the most likely outcome when
180 considering plant-independent effects. Consequently, it was anticipated that any effect
181 of AVG on $CH_4$ oxidation (putatively via reductions in ethylene production) would only
182 manifest without IR, as the IR treatment would make the AVG treatment redundant.
183 However, $CH_4$ oxidation rates in plots treated with the either IR or IR and AVG in
184 combination were not significantly greater than untreated control plots. It is possible
185 that the moisture addition associated with the IR treatment was insufficient to
186 substantially alleviate plant drought stress, driving an increase in ethylene production,
187 which could then account for the numerical difference between the AVG and IR
188 treatments (Fig. 2). The water addition may have also been insufficient to meaningfully
189 and directly stimulate methanotroph activity. However, it would be expected that the
190 combination of IR and AVG would support soil $CH_4$ oxidation rates either the same, or
191 potentially greater than, those observed for AVG alone. This was not the case, and the
192 explanation for the significant interaction remains unknown. As discussed above, it is
193 possible for increased soil moisture content to inhibit $CH_4$ oxidation via decreased
194 porosity and gas diffusion (see Zhou et al., 2014), but given the IR treatment alone did
195 not reduce $CH_4$ oxidation rates relative to the control, this is not a feasible explanation
196 in this case.

197 The lack of data explicitly describing ethylene release into soil in response to the
198 treatments is a limitation to this trial. However, the quantification of ethylene in soil is
199 not trivial, particularly when conducted over time (i.e. continuous) and was outside the
200 resources available for this study. However, given the findings of this study, and
201 considering treatments were field-based, further investigations of the interactions
202 between AVG, plant stress, and $CH_4$ oxidation should be conducted. In these studies,
203 consideration should be given to collection and integration of ethylene data, particularly
204 given that this data may help shed light on the nature of any interactions between
205 treatments.

206 Overall, the findings of this study indicate that application of an ethylene
207 biosynthesis inhibitor to plant tissue can cause a measurable increase in the capability
208 of soil to oxidise $CH_4$ under moisture stressed conditions. This supports the hypothesis

209 that the stress-induced production of ethylene by plants can disrupt the activity of
210 methanotrophs, as well as identifying a potential management pathway to help retain,
211 or even enhance, the methanotrophic capability of soils in productive systems. Given
212 the global importance of a positive feedback between environmental stress, plant
213 ethylene production, and lowered microbial $CH_4$ oxidation activity, further work in this
214 area is needed. In addition, methods to moderate impacts on the methanotrophic
215 community, such as use of alternative forms or rates of biochar application, require
216 investigation to enable provision of important ecosystem services.
217
218 **Acknowledgements** The research was jointly supported by National Natural Science
219 Foundation of China (No. 31600406), Shanghai Science and Technology Innovation
220 Fund (No. 18391902300), Griffith University Research Fellowship, a Collaborative
221 Research Network-the University of the Sunshine Coast Research Futures Project Seed
222 Grant, New Zealand and the "Growing Confidence in Forestry's Future" research
223 programme (C04X1306), which is jointly funded by the Ministry of Business
224 Information and Employment (MBIE) and the Forest Growers Levy Trust, with the
225 support of the NZ Forest Owners Association (FOA) and the NZ Farm Forestry
226 Association (FFA).
227
228 **References**
229 Argueso C.T., Hansen M. and Kieber J.J.: Regulation of ethylene biosynthesis, J Plant Growth
230 Regul., 26, 92-105, 2007.
231 Boller T., Herner R.C. and Kende H.: Assay for and enzymatic formation of an ethylene precursor,
232 1-aminocyclopropane-1-carboxylic acid, Planta 145, 293-303, 1979.
233 Bousquet P., Ciais P., Miller J.B., Dlugokencky E.J., Hauglustaine D.A., Prigent C., van der Werf
234 G.R., Peylin P., Brunke E.G., Carouge C., Langenfelds R.L., Lathière J., Papa F., Ramonet
235 M., Schmidt M., Steele L.P., Tyler S.C. and White J.: Contribution of anthropogenic and
236 natural sources to atmospheric methane variability, Nature, 443, 439-443, 2006.
237 Forster P., Ramaswamy V., Artaxo P., Berntsen T., Betts R., Fahey D.W., Haywood J., Lean J., Lowe
238 D.C., Myhre G., Nganga J., Prinn R., Raga G., Schulz M. and van Dorland R.:Changes in

239 atmospheric constituents and in radiative forcing. In: Solomon S et al. (eds) Climate Change
240 2007: The Physical Science Basis. Cambridge University Press, UK, pp 130–234, 2007.
241 Ho A., Reim A., Kim S.Y., Meima-Franke M., Termorshuizen A., de Boer W., van der Putten W.H.
242 and Bodelier P.L.E.: Unexpected stimulation of soil methane uptake as emergent property
243 of agricultural soils following bio-based residue application, Global Change Biol., 21, 3864-
244 3879, 2015.
245 Isbell R.F.: A classification system for Australian soils (third approximation) CSIRO Australia
246 Division of Soils Technical Report 2/1993, CSIRO Division of Soils and National Landcare
247 Program project, Townsville, 1993.
248 Jäckel U., Schnell S. and Conrad R.: Microbial ethylene production and inhibition of
249 methanotrophic activity in a deciduous forest soil, Soil Biol. Biochem., 36, 835-840, 2004.
250 Karhu K., Mattila T., Bergström I. and Regina K.: Biochar addition to agricultural soil increased
251 $CH_4$ uptake and water holding capacity - Rresults from a short-term pilot field study, Agric.
252 Ecosyst. Environ., 140, 309-313, 2011.
253 Kim J., Yoo G., Kim D., Ding W. and Kang H.: Combined application of biochar and slow-release
254 fertilizer reduces methane emission but enhances rice yield by different mechanisms, Appl.
255 Soil Ecol., 117-118, 57-62, 2017.
256 Kolb S.: The quest for atmospheric methane oxidizers in forest soils, Environ. Microbiol Rep., 1,
257 336–346, 2009.
258 Le Mer J. and Roger P.: Production, oxidation, emission and consumption of methane by soils: A
259 review, Eur. J. Soil Biol., 37, 25-50, 2001.
260 Morgan P.W. and Drew M.C.: Ethylene and plant responses to stress, Physiologia Plantarum, 100,
261 620–630, 1997.
262 Pierik R., Tholen D., Poorter H., Visser E.J.W., Voesenek L.A.C.J.: The Janus face of ethylene:
263 growth inhibition and stimulation, Trends Plant Sci., 11,176–183, 2006.
264 Xu X., Yuan B. and Wei J.: Vertical distribution and interaction of ethylene and methane in
265 temperate volcanic forest soils, Geoderma, 145, 231-237, 2008.
266 Zhou X.Q., Wang Y.F., Huang X.Z., Tian J.Q. and Hao Y.B.: Effect of grazing intensities on the
267 activity and community structure of methane-oxidizing bacteria of grassland soil in Inner
268 Mongolia, Nutr. Cycl. Agroecosys, 80, 145-152, 2008.

Zhou X.Q., Smaill S.J. and Clinton P.W.: Methane oxidation needs less stressed plants, Trends Plant Sci., 18, 657-659, 2013.

Zhou, X.Q., Dong, H.B., Chen, C.R., Smaill, S.J., Clinton, P.W.: Ethylene rather than dissolved organic carbon controls methane uptake in upland soils, Global Change Biol 20, 2379-2380, 2014.

Zhou X.Q., Guo Z.Y., Chen C.R. and Jia Z.J.: Soil microbial community structure and diversity are largely influenced by soil pH and nutrient quality in 78-year-old tree plantations, Biogeosciences, 14, 2101-2111, 2017.

**Table 1**. Analysis of treatment effects on methane oxidation rates, accounting for the split-plot design of the trial.

| ***Block*** | Df | Sum Sq | Mean Sq | F Value | Pr(>F) |
|---|---|---|---|---|---|
| Residuals | 4 | 25.27 | 6.318 | | |
| ***Block:IR*** | Df | Sum Sq | Mean Sq | F Value | Pr(>F) |
| IR | 1 | 0.057 | 0.057 | 0.01 | 0.925 |
| Residuals | 4 | 22.785 | 5.696 | | |
| ***Block:IR:BC*** | Df | Sum Sq | Mean Sq | F Value | Pr(>F) |
| BC | 1 | 0.946 | 0.946 | 0.245 | 0.634 |
| IR:BC | 1 | 0.815 | 0.815 | 0.211 | 0.658 |
| Residuals | 8 | 30.924 | 3.865 | | |
| ***Block:IR:BC:AVG*** | Df | Sum Sq | Mean Sq | F Value | Pr(>F) |
| AVG | 1 | 4.77 | 4.768 | 1.796 | 0.199 |
| IR:AVG | 1 | 17.38 | 17.384 | 6.549 | **0.021** |
| BC:AVG | 1 | 6.19 | 6.186 | 2.33 | 0.146 |
| IR:BC:AVG | 1 | 0.42 | 0.422 | 0.159 | 0.695 |
| Residuals | 16 | 42.47 | 2.654 | | |

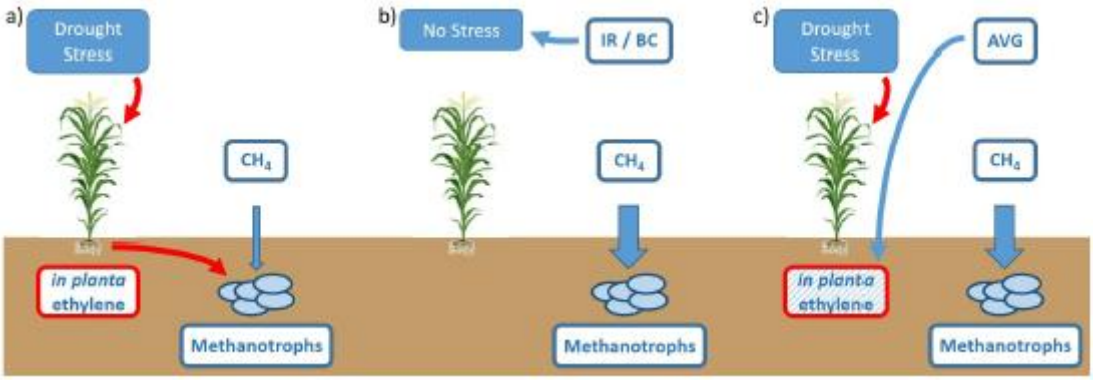


**Fig. 1.** Conceptual outline of the proposed relationships between soil $CH_4$ oxidation rates and aboveground plant biomass with regard to the anticipated effects of the treatments applied in this study. a) Under environmental stress, *in planta* ethylene production is stimulated, resulting in ethylene exudation into the soil atmosphere and the inhibition of soil $CH_4$ oxidation by methanotrophs. b) The application of irrigation (IR) increases soil moisture while the application of biochar (BC) increases soil moisture holding capacity, both acting to reduce plant stress and prevent ethylene exudation into the soil atmosphere. c) The application of AVG disrupts ethylene production, and limiting or preventing the inhibition of $CH_4$ oxidation by the stressed plant.

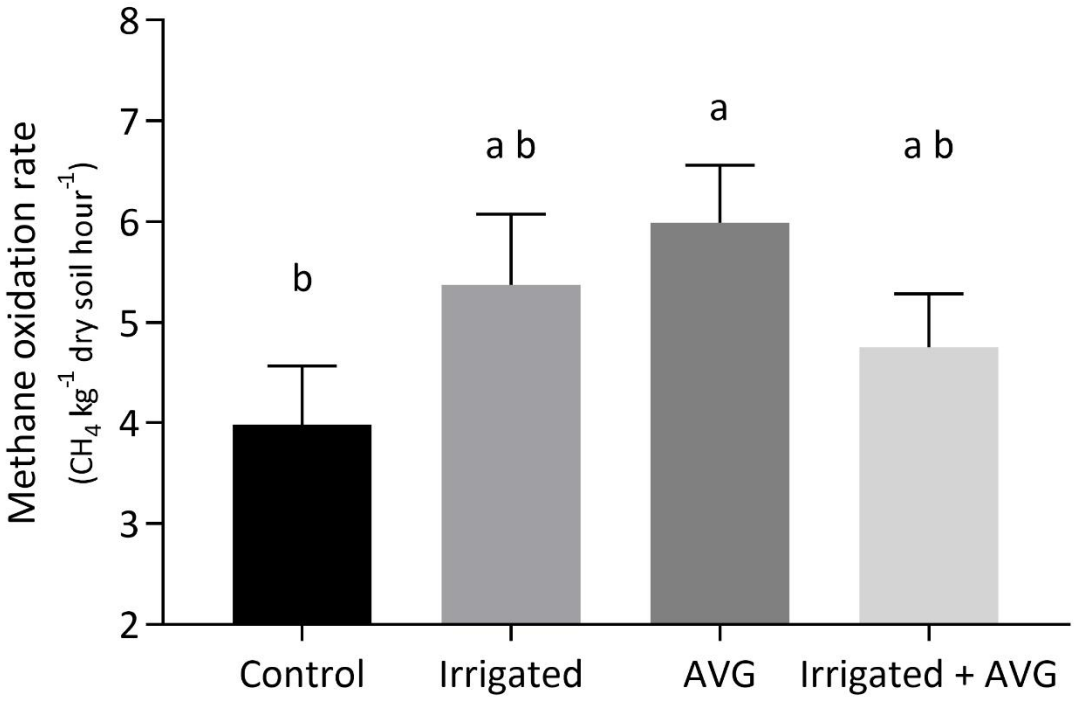


**Fig. 2.** Response of soil $CH_4$ oxidation rates to treatment with irrigation and AVG under maize plants. Letter groupings indicate significant differences at $P<0.05$; error bars are standard error of the mean. The biochar treatment did not influence results, so the data presented are the means of both biochar and no biochar treatments.