

1 **Adaptation to chronic drought modifies soil microbial community responses to phytohormones**

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12 **Abstract**

13 Drought imposes stress on plants and associated soil microbes, inducing coordinated adaptive
14 responses, which can involve plant–soil signalling via phytohormones. However, we know little
15 about how microbial communities respond to phytohormones, or how these responses are shaped
16 by chronic (long-term) drought. Here, we added three phytohormones (abscisic acid, 1-
17 aminocyclopropane-1-carboxylic acid, and jasmonic acid) to soils from long-term (25-year), field-
18 based climate treatments to test the hypothesis that chronic drought alters soil microbial
19 community responses to plant stress signalling. Phytohormone addition increased soil respiration,
20 but this effect was stronger in irrigated than in droughted soils and increased soil respiration at low
21 phytohormone concentrations could not be explained by their use as substrate. Thus, we show that
22 drought adaptation within soil microbial communities modifies their responses to phytohormone
23 inputs. Furthermore, distinct phytohormone-induced shifts in microbial functional groups in
24 droughted vs. irrigated soils might suggest that drought-adapted soil microorganisms perceive
25 phytohormones as stress-signals, allowing them to anticipate impending drought.

26

27 **Introduction**

28 Interactions between plants and soil micro-organisms play a critical role in determining the
29 response of terrestrial ecosystems to a changing climate¹. The establishment and maintenance of
30 relationships between plants and microbes requires mutual recognition of the responses of both
31 partners to changes in their immediate environments^{2,3}, which is largely thought to be mediated by
32 the reciprocal exchange of resources⁴. Plants can rapidly stimulate microbial activity via root
33 exudates and signalling molecules, such as phytohormones⁵ and plants adapted to different climate
34 conditions can modify soil microbial activity by altering the composition of root exudates⁶. Whereas
35 most root inputs function largely as a source of carbon and nutrients for soil microbial communities,
36 phytohormones can act both as substrate and as signalling molecules². Molecular signalling
37 represents an important communication pathway between plants and microbes, whereby plant

38 hormone inputs modify microbial community structure or activity, and microbial metabolism or
39 synthesis of phytohormones enables them to influence plant growth and performance^{7,8,9}. Such
40 bidirectional communication between plants and microorganisms can result in coordinated
41 responses to environmental changes^{9,10}, which will shape overall ecosystem function.
42 Phytohormones can be released into the soil via diffusion or active transport as well as actively
43 exuded by roots^{11,12} and the root exudates of drought-stressed plants show increased
44 concentrations of phytohormones¹³. Whereas numerous studies have investigated soil microbial
45 responses to root exudate compounds such as sugars, amino acids, organic acids^{14,15} or secondary
46 metabolites¹⁶, we still know very little about the influence of phytohormones on soil microbial
47 activity.

48 The role of phytohormones in coordinating plant-microbial responses to drought is of particular
49 interest because plants are immobile, and their survival depends largely upon their ability to rapidly
50 adjust their physiology and growth to mitigate the impacts of drought stress; processes which are
51 usually mediated by phytohormones^{3,17}. Roots in contact with drying soil show altered hormone
52 accumulation^{18,19,20} and increased hormone efflux to the rhizosphere can shape plant-associated
53 microbial communities, which in turn influence plant performance, resulting in coordinated plant-
54 microbial responses to drought^{21,22}. Soil microbial community composition is altered by exogenous
55 application of several phytohormones involved in plant drought responses, including abscisic acid,
56 ethylene, and jasmonates²³. Abscisic acid (ABA) is an important signalling molecule in plants and
57 microorganisms²⁴. ABA is synthesised throughout the plant in response to decreased tissue water
58 status and maintains root growth and hydraulic conductance in drying soil⁷. Water-stressed roots
59 exude ABA into the soil²⁵ where it can be metabolized by some fungi and bacteria²⁶. Drought also
60 stimulates plant production of ethylene, and its precursor 1-aminocyclopropane-1-carboxylic acid
61 (ACC). As ACC usually inhibits root elongation²⁷, bacterial degradation of ACC can stimulate root
62 growth, even under water-stress¹⁷. Finally, although jasmonates such as jasmonic acid (JA) are
63 usually associated with biotic stress²⁸, they also affect plant drought responses by modulating root

64 hydraulic conductance and stomatal closure²⁹. Jasmonates can influence rhizosphere microbial
65 communities indirectly by altering the concentrations of different compounds in root exudates³⁰ and
66 marked shifts in microbial community structure have also been observed in direct response to
67 exogenous application of methyl jasmonate²³. Hence, by modulating plant stress responses and
68 shaping microbial community structure, phytohormones could play a significant role in the co-
69 adaptation of plants and soil microbial communities to drought⁹.

70 Research into plant-hormone mediated microbial activity under drought conditions has largely
71 focussed on rhizosphere or endophytic organisms that are tightly associated with the plant^{31,32,33}. It
72 is well known that plant growth promoting rhizobacteria (PGPR) can interact with or manipulate
73 plant hormone signalling by synthesising or metabolising phytohormones^{7,8}. However, many other
74 naturally occurring soil organisms respond to root efflux of phytohormones², but the role of
75 phytohormones in shaping soil microbial communities and processes more generally has received
76 little attention. Since plant drought responses are mediated by phytohormones, frequent or chronic
77 water-deficit could result in high exposure of soil microbial communities to these stress-signalling
78 molecules. The responses of broad soil microbial communities to root phytohormone efflux from
79 water-stressed plants represents a major gap in our understanding of plant-soil interactions under
80 drought because numerous important ecosystem processes are modulated by microbial community
81 structure and activity in bulk soils¹. Shifts in the relative abundance and dominance of soil micro-
82 organisms in response to drought can be regarded as community-level adaptation, resulting in
83 differentiated microbial communities in droughted vs. non-droughted soils¹⁰, which in turn can
84 modify microbially-mediated soil processes³⁴. In particular, proliferation of slow-growing
85 oligotrophic microbes under drought can enhance community-level drought-tolerance, and the
86 accompanying changes in resource-use strategies alter carbon and nutrient dynamics in soils^{10,35}.
87 Hence, identifying the role of plant stress hormones in shaping microbial communities and activities
88 under drought is an important first step to establish whether molecular signalling enables plant-
89 microbial co-adaptation, which could ultimately shape ecosystem processes.

90 Here, we assessed the effects of three drought-associated phytohormones on soil microbial
91 activity and community structure to investigate whether chronic drought and irrigation treatments
92 influence soil microbial community responses to phytohormones. We used soils from chronic
93 drought and irrigation treatments within the Buxton Climate Change Impacts Study, where
94 temperature and rainfall have been experimentally manipulated since 1993³⁶. Such prolonged
95 treatments (25 years) represent a strong selective pressure on both plants and soil microbial
96 communities^{37,38,39}. Previous work at the study site demonstrated that drought-mediated changes in
97 soil microbial communities were linked to changes in the plant community via shifts in plant traits
98 representing altered resource quality for soil microorganisms^{37,38,40}. Hence, chronic climate
99 treatments have altered soil microbial community structure, and we hypothesised that soil microbial
100 communities from long-term drought plots would respond more strongly to plant stress hormones,
101 compared with soil microbes from irrigated or control plots. To test our hypotheses, we quantified
102 changes in soil microbial activity (respiration rates) in response to different concentrations of ABA,
103 JA, and ACC using a microplate assay, and we assessed changes in soil microbial community
104 structure using phospho-lipid fatty acid (PLFA) biomarkers. We observed increased soil microbial
105 respiration following phytohormone additions, which could not be explained by substrate use alone.
106 Importantly, the magnitude of the respiration response differed among climate treatments and
107 shifts in microbial biomarkers with phytohormone addition differed markedly between soils
108 subjected to long-term drought or irrigation treatments. Thus, our study demonstrates that
109 adaptation to long-term drought modifies the responses of soil microbial communities to plant
110 stress hormones.

111

112 **Results**

113 ***Soil respiration in response to phytohormone additions***

114 Respiration rates in procedural controls without phytohormone addition did not differ among
115 climate treatments and the soils contained similar concentrations of extractable carbon (C) and

116 nitrogen (N; Table 1). However, the respiration rate of all soils increased following phytohormone
117 addition and the magnitude of the response differed among climate treatments, whereby the
118 increases in respiration were generally greater in irrigated soils compared to droughted or control
119 soils (Figure 1). There was no clear trend in respiration responses with increasing phytohormone
120 concentration, but we note that the extra carbon released by increased respiration at nanomolar
121 phytohormone concentrations exceeded the amount of carbon added with the phytohormone
122 solutions (Supplementary Methods 1; Table S1), indicating that microbial activity was triggered by
123 phytohormone inputs at nanomolar concentrations. Furthermore, the effects of phytohormones on
124 soil respiration persisted when they were added in conjunction with root exudate solution
125 (Supplementary Methods 2; Figure S1).

126 Following ABA addition, respiration increased only at the highest concentration (1 mM) and the
127 increase differed among climate treatments (climate x concentration interaction: $\chi^2 = 58.9$, $p <$
128 0.001 ; Figure 1a), with a significantly greater respiration response in the irrigated soils than the
129 controls ($p < 0.001$) and droughted soils ($p = 0.001$). Although the increase in respiration from the
130 control soils was not significant ($d = 0.8$; $p = 0.11$), respiration from droughted soils doubled in
131 response to the highest concentration of ABA ($d = 1.95$; $p = 0.006$) and increased more than five-fold
132 in the irrigated soils ($d = 2.27$; $p < 0.001$). Hence, the respiration response to ABA was strongest in
133 irrigated soils.

134 ACC addition also stimulated respiration, and the response at different concentrations was
135 similar among treatments (climate + concentration effect; $\chi^2 = 18.1$, $p = 0.003$; Figure 1b).
136 Respiration rates increased at ACC concentrations of 1 mM ($p = 0.015$) and 1 nM ($p = 0.013$), but not
137 at 1 μ M. The increase in respiration from the control soils in response to ACC was negligible ($d <$
138 0.3), whereas respiration from droughted soils was 1.5x higher than the procedural controls ($d =$
139 1.42 and $d = 0.80$ for 1 mM and 1 nM, respectively), and respiration from the irrigated soils was
140 twice as high ($d = 1.10$ and $d = 0.99$ for 1 mM and 1 nM, respectively). Thus, soil respiration showed
141 an unusual response to ACC addition, which was more pronounced in irrigated soils.

142 Both high and low concentrations of JA were associated with increased respiration rates, but the
143 intermediate concentration was not (climate + concentration effect: $\chi^2 = 13.5$, $p = 0.019$; Figure 1c).
144 Surprisingly, the lowest concentration of JA (1 nM) had the largest effect on respiration in droughted
145 and control soils ($p = 0.006$, $d = 0.5$, 0.1 and 0.11 for control, droughted and irrigated soils,
146 respectively), whereas the increase in respiration at the highest concentration was only apparent in
147 the irrigated soils (1 mM, $p = 0.033$, $d = 3.1$) and there was no effect at the intermediate
148 concentration of JA (1 μ M). Thus, the lowest concentration of JA stimulated respiration regardless of
149 climate treatment.

150

151 ***Shifts in biomarker functional groups***

152 Total pre-incubation phospholipid fatty acid (PLFA) biomass was 21% higher in the irrigated plots
153 compared to the controls and 32% higher than the droughted plots ($F_{2,8} = 11.2$, $p = 0.005$; Table 1).
154 Total biomass increased in all soils during incubation but was unaffected by phytohormone addition.
155 Actinomycete biomarkers did not differ among climate treatments, did not change during the
156 incubation and were also unaffected by phytohormone addition. However, we observed changes in
157 the relative abundance of biomarker functional groups in response to phytohormone addition,
158 which differed among climate treatments, indicated by significant hormone \times climate interactions
159 for biomarkers representing Gram-positive bacteria ($\chi^2 = 25.6$, $p = 0.002$), Gram-negative bacteria
160 ($\chi^2 = 31.2$, $p = 0.001$), the ratio of Gram-positive to Gram-negative biomarkers (G+:G- ratio; $\chi^2 = 32.2$,
161 $p < 0.001$), saprophytic fungi ($\chi^2 = 42.1$, $p < 0.001$), and arbuscular mycorrhizal (AM) fungi ($\chi^2 = 53.6$,
162 $p < 0.001$).

163 The relative abundance of Gram-positive and Gram-negative biomarkers did not differ among
164 treatments before incubation (Table 1) but responded differentially to phytohormone additions. The
165 abundance of Gram-positive biomarkers remained unchanged in control soils but increased
166 significantly in irrigated soils with the addition of ABA and JA, and there was a trend towards
167 increased Gram-positive biomarkers with ACC. By contrast, in droughted soils Gram-positive

168 biomarker abundance declined significantly with the addition of all three hormones (Figure 2a).
169 Gram-negative biomarker abundance remained unchanged in droughted soils but increased with
170 ABA, ACC and JA addition to control soils, and with ACC addition to irrigated soils (Figure 2b). Thus,
171 in response to phytohormone addition, the relative abundance of Gram-positive biomarkers
172 increased in irrigated soils but declined in droughted soils.

173 The G+:G- ratio before incubation was similar among climate treatments (Table 1) but largely
174 followed the response of Gram-positive biomarkers to incubation with phytohormones. The G+:G-
175 ratio increased with addition of ABA and JA in irrigated soils, but declined in control soils with ACC
176 addition and declined in droughted soils in response to all hormones (Figure 2c).

177 Fungal biomass before incubation was 21% lower in droughted plots and 24% higher in the
178 irrigated plots compared to the controls ($F_{6,8} = 19.03$, $p < 0.001$; Table 1). Fungal biomass increased
179 in all climate treatments during incubation but there was no effect of phytohormone addition.
180 Similarly, the ratio of fungi to bacteria before incubation (F:B ratio) was lower in the droughted plots
181 than the irrigated plots or controls ($F_{2,8} = 12.6$, $p = 0.003$; Table 1) but phytohormone addition did
182 not affect the F:B ratio in any climate treatment.

183 The abundance of saprophytic fungal biomarkers before incubation was similar among
184 treatments (Table 1) but declined significantly in control soils in response to ABA and JA, and there
185 was a trend towards a decline in fungal biomarkers with ACC addition. The decline in saprophytic
186 fungal biomarkers with phytohormone addition was even stronger in irrigated soils, with
187 significantly lower abundance in response to all three hormone treatments. By contrast, saprophytic
188 fungal biomarker abundance in the droughted soils increased in response to all three hormone
189 treatments (Figure 3). Thus, in response to phytohormone addition, the relative abundance of
190 saprophytic fungal biomarkers declined in irrigated soils but increased in droughted soils. The
191 abundance of AM fungal biomarkers did not differ among treatments before incubation (Table 1)
192 but AM fungal biomarkers increased significantly in the control soils with addition of all three
193 hormones, and in droughted soils with ABA addition. By contrast, addition of all three hormones

194 significantly decreased AM fungal biomarker abundance in the irrigated soils. Thus, in response to
195 phytohormone addition, the relative abundance of AM fungal biomarkers increased in the control
196 and droughted soils but declined in the irrigated soils (Figure 3b).

197

198 **Discussion**

199 Our study demonstrates that community-level adaptation to chronic climate change modifies the
200 response of soil microbes to phytohormone inputs. Phytohormone addition clearly stimulated soil
201 microbial activity, but the magnitude of the effects differed for soil microbial communities
202 conditioned under different climate treatments at Buxton. The increased respiration rates at low
203 concentrations of phytohormones and the contrasting shifts in microbial functional groups in
204 droughted vs. irrigated soils are particularly intriguing. Here, we explore the possibility that soil
205 microbial communities might respond to phytohormones as signals of impending water-stress,
206 rather than as substrates for growth.

207 Increased respiration rates in response to phytohormones could indicate that bulk soil
208 microorganisms can utilise all three hormones as substrates, and the greater respiration response to
209 phytohormones in the irrigated compared to droughted soils strongly suggests community-level
210 adaptation of soil microorganisms to field conditions. Microbial community-level adaptation to
211 drought often involves shifts towards organisms with greater tolerance to water deficit, as well as
212 increased abundance of slow-growing taxa with conservative resource-use strategies^{10,41}. Distinct
213 resource-use efficiencies of the microbial communities would explain some of the differences in the
214 respiration responses to phytohormones, despite similar concentrations of readily available soil C
215 and N among climate treatments (Table 1). In the irrigated soils, the substantial increases in
216 respiration following phytohormone addition are characteristic of a microbial community dominated
217 by copiotrophic organisms that preferentially use labile substrate and can rapidly respond to
218 changes in resource inputs¹⁰. In droughted soils, smaller respiration responses to phytohormones
219 correspond to a stress-tolerant microbial community dominated by taxa with reduced metabolic

220 capacity⁴¹. By contrast, the control plots at our study site experience large natural fluctuations in soil
221 moisture³⁸ resulting in microbial communities composed of taxa with broad tolerances³⁴, which is
222 reflected in the variable respiration rates in control soils both with and without phytohormone
223 addition (Figure 1). Hence, highly differentiated microbial communities in soils sampled several
224 months after the end of the summer drought treatment at Buxton (Supplementary Methods 3,
225 Figure S2) indicate lasting shifts in microbial community structure that likely underpin the distinct
226 respiration responses in our experiment.

227 Three lines of evidence indicate that microbial utilisation of phytohormones as substrate cannot
228 fully explain our findings. First, if increased respiration rates were solely due to microbial utilisation
229 of phytohormones as substrates, we would expect the highest respiration rates at the highest
230 phytohormone concentrations in all climate treatments, but this was only observed for ABA (Figure
231 1a). Thus, the consistent response to ABA at high concentrations likely reflects microbial utilisation
232 of ABA as substrate²⁶. However, the inconsistent response to ACC addition is surprising because
233 numerous microorganisms can also utilise ACC as a source of C and N⁷. Second, the effects of
234 phytohormones on soil respiration persisted even when they were added in conjunction with root
235 exudate solution, which provides an ample source of C and N to soil microbes (Table S2; Figure S1).
236 Finally, microbial use of phytohormones as substrate cannot explain the increased respiration at the
237 lowest concentrations of JA and ACC, where the additional release of carbon as CO₂ exceeded C
238 inputs (Supplementary Methods 1, Table S1). Such a disproportionate increase in respiration in
239 response to very small resource inputs indicates that JA and ACC stimulated microbial mineralisation
240 of existing soil C, a phenomenon previously demonstrated for other organic substances contained in
241 root exudates^{14,15,42,43}. Hence, whereas increased respiration at high (millimolar) concentrations of all
242 phytohormones likely reflects their utilisation by microbes as C and nutrient sources, increased
243 respiration following ACC and JA addition at nanomolar concentrations suggests that these
244 phytohormones can also trigger microbial activity, and the extent of the response clearly differed
245 among climate treatments. Whereas increased respiration with 1 nM ACC might be explained by

246 promotion of fungal development at low concentrations ($< 1 \text{ mM}$)⁴⁴, the respiration response of
247 droughted and irrigated soils to 1 nM JA is particularly intriguing because plant development
248 appears to benefit more at low concentrations of jasmonates than at high concentrations⁴⁵ but the
249 microbial metabolic pathways for JA are largely unknown⁴⁶. Although it is conceivable that increased
250 respiration at low phytohormone concentrations could be entirely explained by ‘priming’ of soil C⁴²,
251 such priming did not occur in response to adding a standard root exudate solution, and respiration
252 rates with the root exudate solution did not differ among climate treatments (Supplementary
253 Methods 2; Figure S1). Hence, although we are unable to identify mechanisms, we propose that
254 climate-driven adaptation of soil microbial communities not only alters their capacity to utilise
255 phytohormones as a substrate but might also modulate their responses to plant stress hormones as
256 signalling molecules.

257 The contrasting effects of phytohormones on biomarker abundances in droughted vs. irrigated
258 soils provide further strong evidence for community-level microbial adaptation to drought. As
259 microbial biomass was unaffected by phytohormone addition, the observed changes in the relative
260 abundances of biomarker functional groups in response to phytohormones likely represent shifts in
261 dominance, activity, or turnover, rather than growth in overall community size⁴⁷. The disparate
262 response of fungal biomarkers in droughted vs. irrigated soils is particularly striking (Figure 3)
263 because fungi are inherently more drought-resistant than bacteria^{41,48}, whereas bacteria grow faster
264 and outcompete fungi for access to labile substrates⁴⁹ such as phytohormones. However, if chronic
265 drought has selected against fast-growing stress-intolerant bacteria¹⁰, a greater proportion of
266 resources might be available to fungi. Previous work at our study site demonstrated large changes in
267 fungal community structure and the absence of *c.* 25% of fungal taxa in the droughted plots³⁸, as
268 well as shifts in mycorrhizal colonization rates and extraradical hyphal abundance⁵⁰, suggesting that
269 this chronic treatment has selected against drought-sensitive fungi. A more stress-tolerant fungal
270 community in droughted soils, combined with resource competition between drought-adapted fungi

271 and bacteria could explain why fungal abundance increased following phytohormone application in
272 droughted soils, but declined in the controls and irrigated soils.

273 Many Gram-negative bacteria are fast-growing organisms⁵¹, and a wide range of Gram-negative
274 genera can metabolise ACC^{7,52}, so it is noteworthy that the largest increases in Gram-negative
275 biomarker abundance occurred after adding ACC to irrigated and control soils. Conversely, selection
276 against fast-growing opportunistic bacteria would explain why Gram-negative bacteria did not
277 respond to any hormone additions in the droughted soils, as chronic drought would favour stress-
278 tolerant, slow-growing Gram-negative taxa with thick cell-walls that are more similar to Gram-
279 positive physiology^{10,53,54}. At first glance, it seems surprising that the abundance of Gram-positive
280 biomarkers in the droughted soils declined following phytohormone addition (Figure 2a), because
281 they are generally assumed to be more stress-tolerant than Gram-negative bacteria^{41,48}. However,
282 we speculate that the declines in Gram-positive biomarker abundance following addition of all three
283 phytohormones indicate drought-adapted bacteria that respond negatively to stress signalling,
284 possibly by investing resources in survival strategies such as dormancy, osmolytes or spore
285 production instead of growth and turnover^{41,48,55}.

286 The contrasting responses of Gram-positive biomarker abundance following phytohormone
287 additions to droughted vs. irrigated soils raise the intriguing possibility that drought-adapted soil
288 microbial communities might perceive these phytohormones as signals of impending water-stress,
289 rather than as substrate for growth. Phytohormones could act as early-warning signals for soil
290 microbial communities because root hormone accumulation in relatively moist soil ($\Psi < -0.1$ MPa⁵⁶)
291 indicates that plants detect soil water deficits at much higher bulk soil water potentials than bacteria
292 ($\Psi < -1.0$ MPa⁵⁷). If phytohormones activate acquired microbial stress responses, they would allow
293 microbes to improve defences against future stress⁵⁸. As drought stress responses such as spore
294 formation or solute accumulation are energetically costly⁴¹, it is possible that some of the increased
295 respiration in response to phytohormones in the droughted soils reflects enhanced metabolic
296 demands of micro-organisms as they activate stress responses^{58,59}. However, we note that although

297 increased respiration in soils from different climate treatments might be attributed to distinct
298 mechanisms, our measurements cannot distinguish between substrate-induced respiration and
299 respiration as a result of other microbial metabolic activities. Hence, we propose an emerging
300 hypothesis, whereby phytohormones induce adaptive microbial responses or defences to impending
301 water-stress, and chronic drought selects for microorganisms that respond to stress signalling by
302 entering dormancy, or producing spores or extra-cellular polymeric substances, rather than
303 increasing growth and abundance^{41,48,55}. Clearly, our experiment cannot identify the metabolic
304 pathways involved in microbial responses to plant stress signalling. However, our findings highlight
305 an intriguing new avenue for research to identify the mechanisms underpinning the coordinated
306 plant-microbial responses to drought.

307 In summary, we demonstrate that chronic drought modifies the response of soil microbial
308 communities to phytohormones associated with plant water deficit. Differences in the structure and
309 metabolic activity of the soil microbial communities in droughted vs. irrigated soils suggest
310 community-level adaptation to the long-term climate treatments, which shaped the response of the
311 microbial communities to phytohormone additions. Given the divergent responses of both microbial
312 activity and biomarker abundances in droughted vs. irrigated soils, we call for targeted studies to
313 address our hypothesis that drought-adapted microbial communities might use phytohormones as
314 signals of impending water stress. The capacity of soil microbial communities to respond to plant
315 stress-signalling by modulating their metabolic activity could affect numerous microbial processes
316 underpinning ecosystem nutrient and carbon dynamics. Consequently, understanding how
317 phytohormones mediate plant-microbial responses to stress could provide new opportunities to
318 enhance the resistance of ecosystem processes to drought. Although we measured respiration rates
319 as a key response of the soil microbial community, adaptation to drought is often characterised by
320 increased abundance of specialist organisms¹⁰, which could affect other important soil processes or
321 influence plant performance. Our findings therefore represent an important step towards identifying

322 how root phytohormone efflux by plants drives selection for drought-resistant soil microbial
323 communities, which will advance our understanding of ecosystem responses to climate change.

324

325 **Methods**

326 ***Study system and field sampling***

327 The Buxton Climate Change Impacts Study (henceforth “Buxton”) is located on calcareous
328 grassland in Derbyshire, UK. The climate treatments have been applied to 3-m × 3-m plots in five
329 fully randomized blocks since 1993^{36,60}. We sampled soils from three climate treatments: summer
330 drought, in which rainfall is excluded using automated shelters from July-August (‘droughted’);
331 supplemented rainfall to 20% above the long-term average from June-September (‘irrigated’); and
332 control plots that experience the ambient climate (‘controls’). Samples were collected in October
333 2018, when soil water content in the droughted plots had recovered to control levels (Table 1).
334 Three cores were taken to 10-cm depth in each of five replicate plots per climate treatment using a
335 1-cm diameter punch corer. The cores were homogenised to give one composite sample per plot,
336 sieved (2-mm) to remove stones and debris, and stored at 4°C for five days. We used a 5-g
337 subsample of each soil to determine extractable carbon (C) and nitrogen (N) as a measure of easily
338 available resources to soil microbial communities⁶¹. The subsamples were extracted in 40 ml 0.5M
339 potassium sulphate (K₂SO₄) solution, shaken for 1 h, filtered through pre-washed filter paper, and
340 the total C and N content of the extracts were analysed by oxidation combustion (TOC-L, Shimadzu
341 Corporation, Kyoto, Japan).

342 ***Phytohormone assays***

343 To assess the influence of phytohormones on soil microbial activity, we measured soil respiration
344 using MicrorespTM, a colourimetric microplate method that measures CO₂ efflux from a sample via an
345 indicator dye (cresol red) suspended in agar that changes colour when CO₂ reacts with bicarbonate
346 in the agar gel⁶². Solutions of abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), and
347 jasmonic acid (JA) were each prepared in concentrations of 1 mM, 1 μM and 1 nM. Whereas

348 nanomolar concentrations of phytohormones are realistically found in soils⁷, millimolar
349 concentrations are applied in horticulture as plant growth regulators^{63,64} and are relevant as
350 potential substrates for microbes, and 1 μ M was included as an intermediate concentration. The
351 phytohormone solutions were compared to procedural controls with deionised water (dH₂O) only,
352 giving ten phytohormone treatments in total. Three analytical replicates were measured for each
353 sample and phytohormone treatment, giving a total of 450 micro-incubations (5 replicates \times 3
354 climate treatments \times 10 hormone treatments \times 3 analytical replicates).

355 For the micro-incubations, soils were brought to 40% field capacity and 0.35 g of soil was added
356 to each 1.2-ml well of a 96-deepwell plate (MicroResp, Aberdeen, UK), which was then pre-
357 incubated at 20°C for four days; samples were fully randomised within each plate. Detection plates
358 (MicroResp, Aberdeen, UK) contained 150 μ l 1% agar with 12.5 ppm cresol red indicator dye, 150
359 mM potassium chloride and 2.5 mM bicarbonate, and initial absorbance was measured on a
360 microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) at 570 nm to provide a
361 baseline value (Creamer et al. 2009). After pre-incubation of the soils, 25- μ l aliquots of the
362 phytohormone solutions or deionised water were applied and the detection plate was attached
363 using an airtight silicone seal. The plates were incubated for 6 h at 20°C, after which the colour
364 change in the detection plate was measured at 570 nm to calculate respiration rates (measured
365 absorbance rates minus absorbance for 18 blanks per plate⁶⁵). We determined the calibration curve
366 for absorbance by equilibrating dye solutions at different CO₂ concentrations prepared with
367 standard gas mixtures (0-5% CO₂)⁶⁵, and verified the micro-incubations in two trials using soils from
368 the same field plots: Trial 1 was a pilot test of the microplate method to determine appropriate
369 incubations times and indicator gel sensitivity (Supplementary Methods 4; Figures S3 and S4),
370 whereas Trial 2 used microcosms with larger quantities of soil and direct measurements of CO₂
371 efflux rates (Supplementary Methods 4; Figure S5). We further assessed whether the effects of
372 phytohormone addition persisted when added in conjunction with a standard root exudate solution,

373 which represents a readily available source of carbon and nutrients to soil microbes (Supplementary
374 Methods 2; Table S2).

375 ***Microbial functional groups***

376 The relative abundance of soil microbial functional groups in the soil samples was determined by
377 phospholipid fatty acid (PLFA) analysis. For each soil sample, six subsamples (5 g fresh weight) were
378 weighed into 50 ml tubes and pre-incubated for four days at 20°C. One subsample was then frozen
379 at -80°C (T₀ controls) and the other soils received 360 µl of ABA, ACC, or JA solution at the highest
380 concentration (1 mM), with dH₂O as a procedural control, resulting in 60 incubations (3 climate
381 treatments × 4 hormone treatments × 5 replicates). All samples were incubated for 24 h at 20°C,
382 then frozen at -80°C before being freeze-dried. Phospho-lipid fatty acids (PLFAs) were extracted
383 from all incubations and the unincubated (T₀) soils using c. 1 g freeze-dried soil following USDA
384 protocols⁶⁶ for a high-throughput method⁶⁷. Extracts were analysed by gas chromatography (Agilent
385 Series II 6890, Palo Alto, USA) and peaks were identified using the Sherlock 6.2™ Microbial
386 Identification System (MIDI, Newark, DE, USA).

387 Our assumption of differentiated microbial communities in the climate treatments was verified
388 by non-metric multidimensional scaling based on Bray-Curtis dissimilarities among PLFA biomarkers
389 in unincubated soils (Supplementary Methods 3; Figure S2). The total biomass of all PLFA biomarkers
390 was used as an estimate of active microbial biomass⁶⁸, and PLFA biomarkers representing Gram-
391 positive and Gram-negative bacteria, saprophytic fungi, arbuscular mycorrhizal (AM) fungi, and
392 actinomycetes were used to assess changes in the relative abundances of each functional group
393 (henceforth 'biomarker functional groups'; Supplementary Methods 5; Table S3). The ratios of fungal
394 to bacterial biomarkers (F:B ratio) and Gram-positive to Gram-negative biomarkers (G+:G- ratio)
395 were calculated as additional indicators of change in microbial community structure^{69,70}.

396

397 ***Statistics and reproducibility***

398 Mean values for soil respiration (CO₂ efflux) were calculated from the three analytical replicates
399 and all analyses were conducted using $n = 5$ replicate plots per climate treatment at Buxton. All data
400 were analysed in R version 3.4.0⁷¹ using the lme4 package for linear mixed effects models (LMEs⁷²).
401 We assessed the effect of phytohormone addition on soil respiration for each hormone with
402 separate LMEs, fitting concentration (1 mM, 1 μ M, 1 nM and 0 M), climate treatment, and their
403 interaction as fixed effects and block as a random effect. To compare the magnitude of changes in
404 soil respiration in response to phytohormone additions among climate treatments, we calculated an
405 effect size for each hormone and climate treatment (Cohen's d ⁷³), representing standard deviations
406 of difference based on the change in respiration rates relative to the procedural controls. As initial
407 microbial biomass differed among climate treatments (Table 1), we also analysed the effects of
408 phytohormones on soil CO₂ efflux expressed as the specific respiration rate per unit microbial
409 biomass to indicate differences in microbial metabolic activity⁷⁴, which revealed similar responses to
410 phytohormone additions (Supplementary Methods 6; Figure S6).

411 We assessed differences among treatments in pre-incubation microbial biomass, fungal biomass,
412 microbial biomarkers, and extractable C and N using one-way ANOVAs (*aov* function) with block
413 included as an error term. Changes in microbial functional groups in response to phytohormone
414 addition were then assessed using LMEs for each biomarker group with hormone, climate
415 treatment, and their interaction fitted as fixed effects, and block as a random effect.

416 All LMEs were simplified by sequential removal of terms, comparing models with AIC and p -
417 values. The best models were compared to appropriate null models using likelihood ratio tests and
418 the final model fit was assessed with diagnostic plots⁷⁵. We give Chi-squared and p -values for the
419 comparisons against null models for the final model, and p -values for post-hoc treatment contrasts
420 generated using Satterthwaite's approximation (*diffsmeans* function in the lmerTest package⁷⁶). We
421 report statistically significant effects at $p < 0.05$, and non-significant trends at $p < 0.1$.

422

423 **Data availability**

424 The soil respiration and microbial biomarker data that support the findings of this study are available
425 in figshare⁷⁷ with the identifier DOI 10.6084/m9.figshare.14130065

426

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612

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621

622 **Author contributions**

623 EJS and ICD conceived the study; EJS, ICD and RW wrote the manuscript; APA managed the field site
624 and implemented the climate treatments; JAC developed the lab methodology, conducted the lab
625 work and collected samples with JE and CZH, who also contributed additional data. EJS analysed the

626 data and interpreted the findings with RW and ICD, and all authors commented on the text and
627 contributed to the interpretation of the results.

628

629 **Competing interests**

630 The authors declare no competing interests.

631

632

633 **Table 1. Soil properties and microbial biomarker groups in soils from chronic climate change**
634 **treatments.** Soil water content, extractable soil carbon (C) and nitrogen (N), active microbial
635 biomass derived from phospholipid fatty acid (PLFA) analysis (Total PLFA biomass), and PLFA
636 biomarkers representing microbial functional groups in soils collected from droughted, irrigated and
637 control plots in the Buxton Climate Change Impacts Study; G+ is Gram-positive bacteria, G- is Gram-
638 negative bacteria, F:B is the ratio of fungal to bacterial biomarkers, and AMF is arbuscular
639 mycorrhizal fungi; means \pm standard errors are given for $n = 5$ replicates per climate treatment and
640 different superscript letters denote significant differences among treatments at $p < 0.05$.

	Droughted	Control	Irrigated
Soil water content (%)	32.6 \pm 1.1 ^a	32.7 \pm 1.2 ^a	41.6 \pm 3.3 ^b
Extractable soil C (mg g ⁻¹)	282 \pm 20	313 \pm 29	325 \pm 27
Extractable soil N (mg g ⁻¹)	48.6 \pm 4.7	54.4 \pm 7.2	46.8 \pm 3.5
Total PLFA biomass (nM g ⁻¹)	549 \pm 39 ^a	601 \pm 54 ^a	725 \pm 48 ^b
Fungal biomass (nM g ⁻¹)	80 \pm 5.7 ^b	101 \pm 7.1 ^a	125 \pm 7.3 ^c
G- biomarkers(%)	45 \pm 0.4	45 \pm 0.5	46 \pm 0.4
G+ biomarkers (%)	22 \pm 0.4	22 \pm 0.6	22 \pm 0.2
Fungal biomarkers (%)	14 \pm 1.1	14 \pm 0.9	14 \pm 0.8
AMF biomarkers (%)	4.5 \pm 0.1	4.5 \pm 0.1	4.6 \pm 0.1
Actinomycete biomarkers (%)	11.8 \pm 0.6	11.6 \pm 0.6	11.4 \pm 0.5
G+:G- ratio	0.48 \pm 0.01	0.48 \pm 0.01	0.47 \pm 0.01
F:B ratio	0.26 \pm 0.004 ^b	0.32 \pm 0.01 ^a	0.32 \pm 0.01 ^a

641

642

643 **Figure legends**

644 **Figure 1. Respiration rates (CO₂ efflux) following phytohormone additions to soils from long-term**
645 **climate change treatments.** Soils collected from control, droughted and irrigated plots in the Buxton
646 Climate Change Impacts Study were incubated for 6 h with addition of **a)** abscisic acid (ABA), **b)** 1-
647 aminocyclopropane-1-carboxylic acid (ACC), or **c)** jasmonic acid (JA) at three concentrations
648 compared to procedural controls (concentration = 0 M); boxes denote the 25th and 75th percentiles
649 and median lines are given for $n = 5$ replicates based on independent field plots; whiskers indicate
650 values up to $1.5 \times$ the interquartile range, and dots indicate outliers.

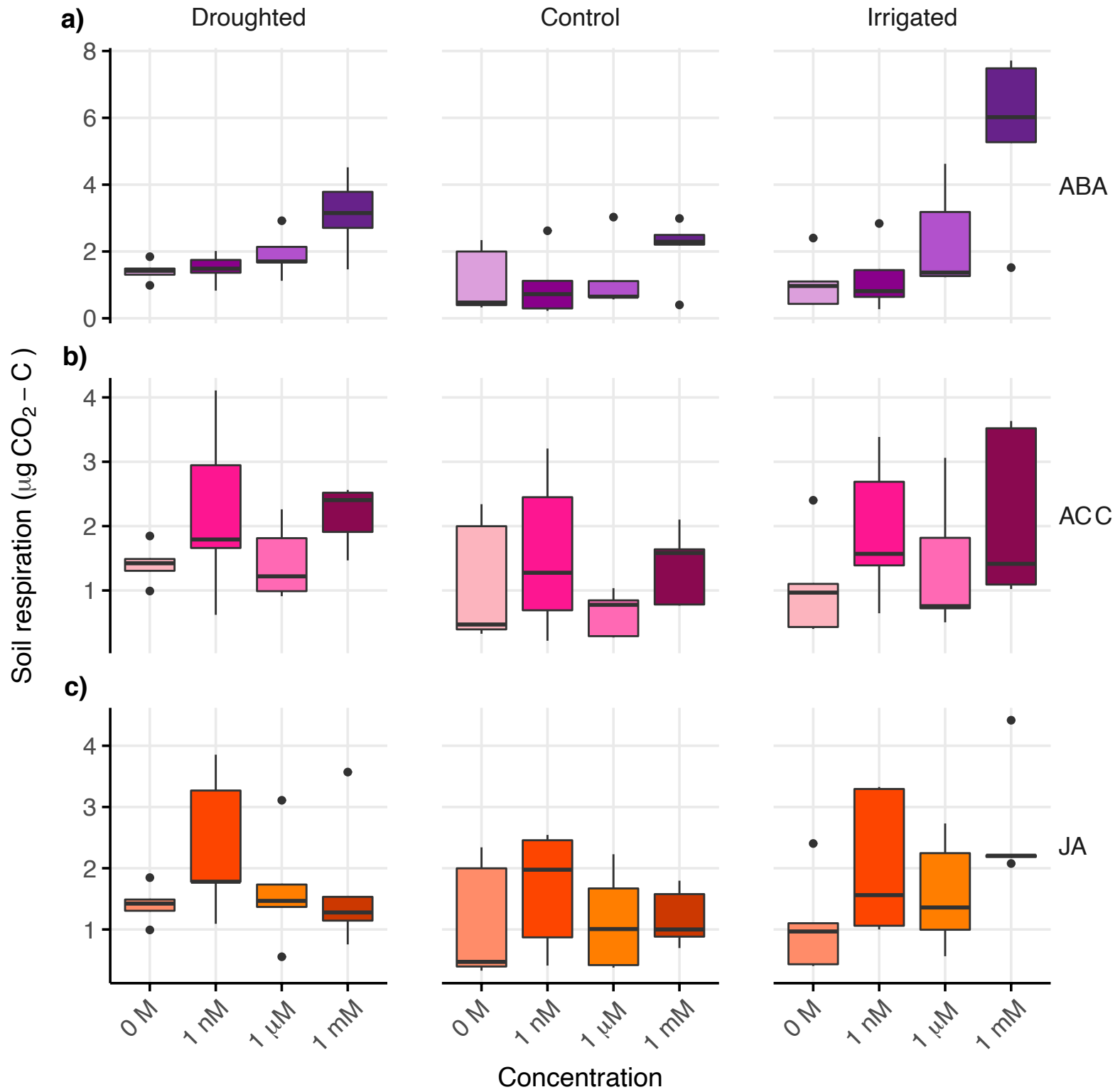
651

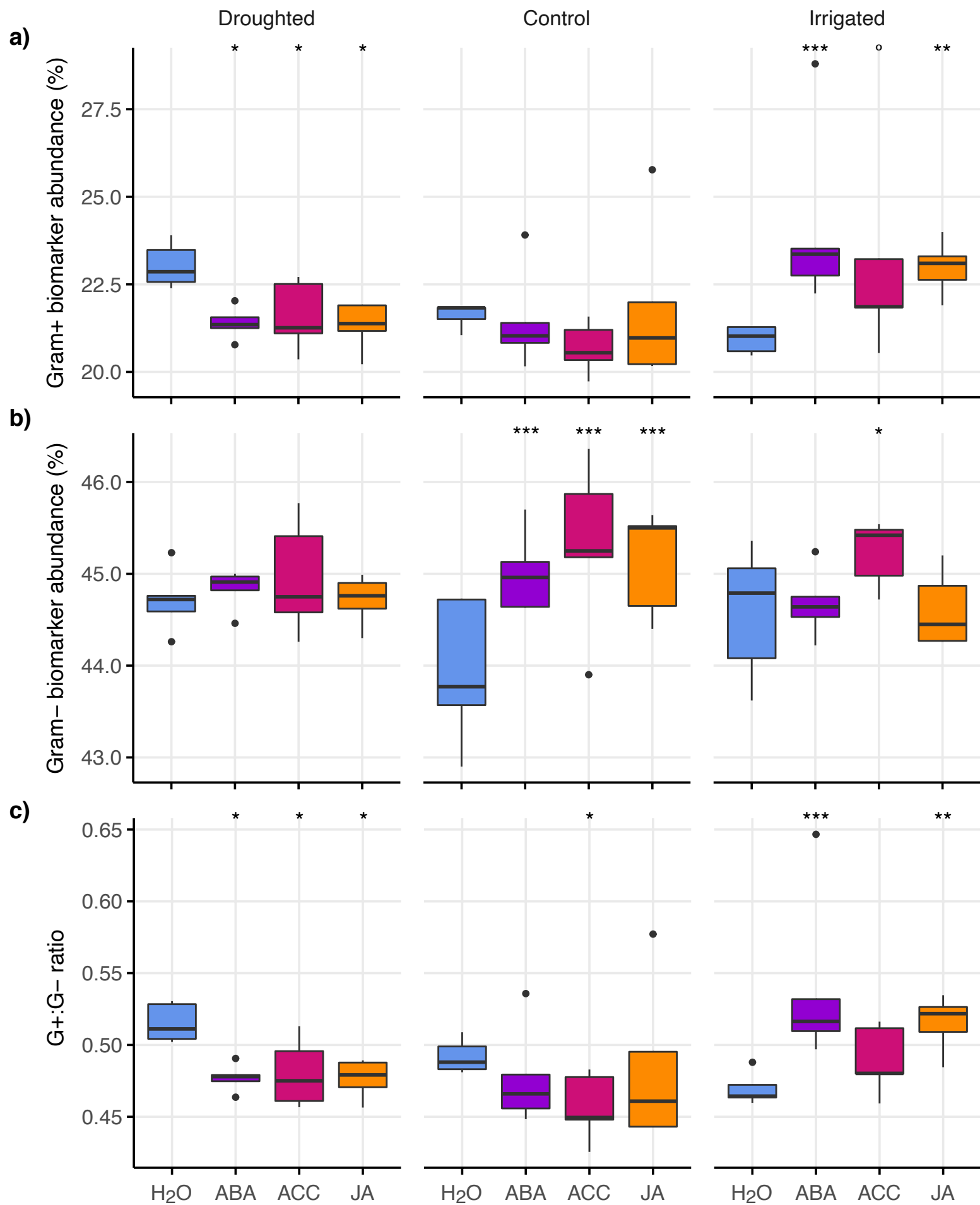
652 **Figure 2. Bacterial biomarker functional groups following phytohormone additions to soils from**
653 **long-term climate change treatments.** Relative abundances of **a)** Gram-positive (Gram+) and **b)**
654 Gram-negative (Gram-) bacteria and **c)** the ratio of Gram-positive to Gram-negative bacteria (G+:G-
655 ratio) were measured in soils collected from control, droughted and irrigated plots in the Buxton
656 Climate Change Impacts Study and incubated for 24 h with abscisic acid (ABA), 1-
657 aminocyclopropane-1-carboxylic acid (ACC), jasmonic acid (JA), or procedural controls (H₂O); boxes
658 denote the 25th and 75th percentiles and median lines are given for $n = 5$ replicates based on
659 independent field plots; whiskers indicate values up to $1.5 \times$ the interquartile range, and dots
660 indicate outliers; effects of hormone application within climate treatments are shown as *** for $p <$
661 0.001; ** for $p < 0.01$, * for $p < 0.05$ and ^o for trends at $p < 0.1$.

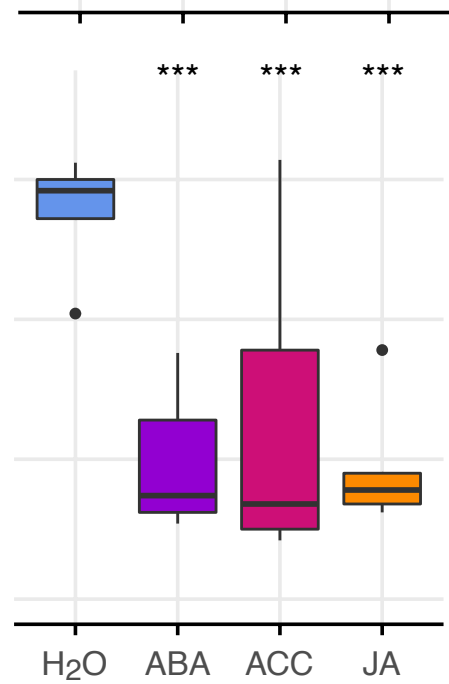
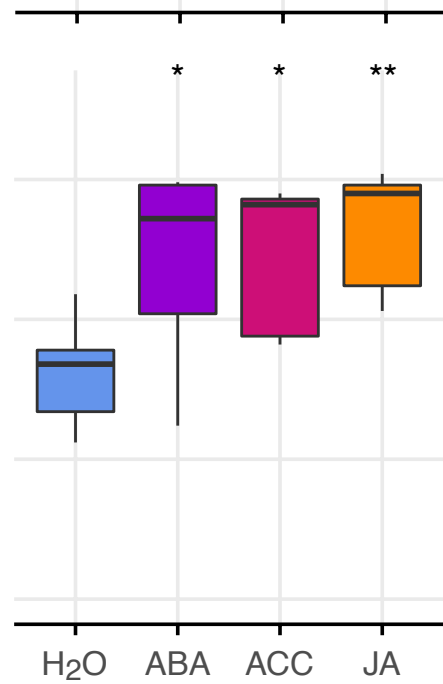
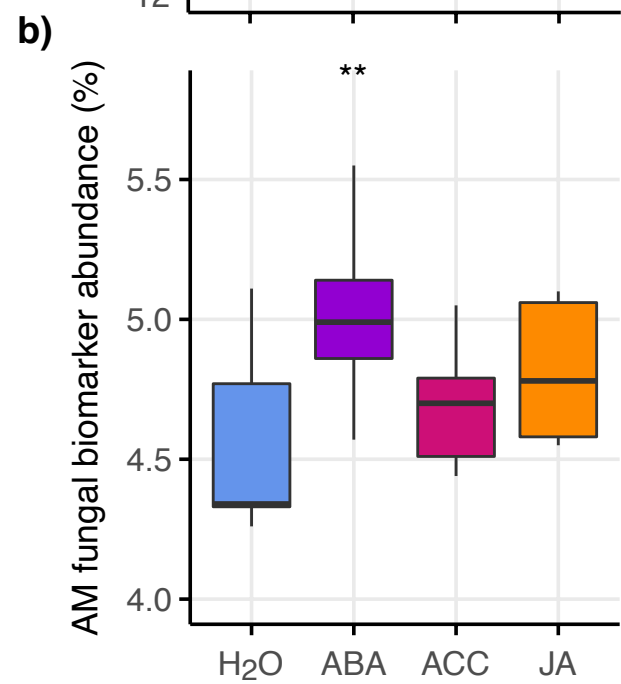
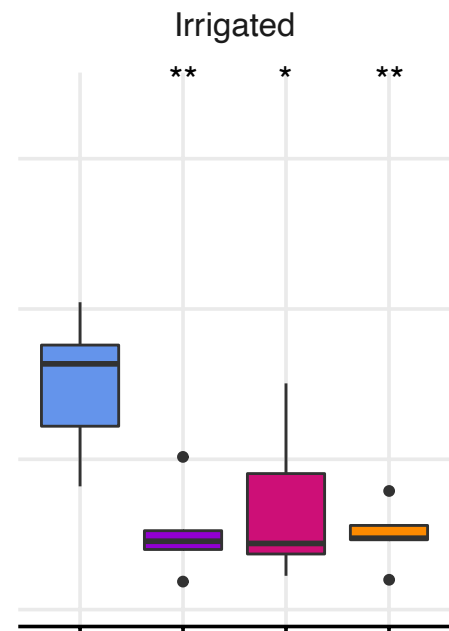
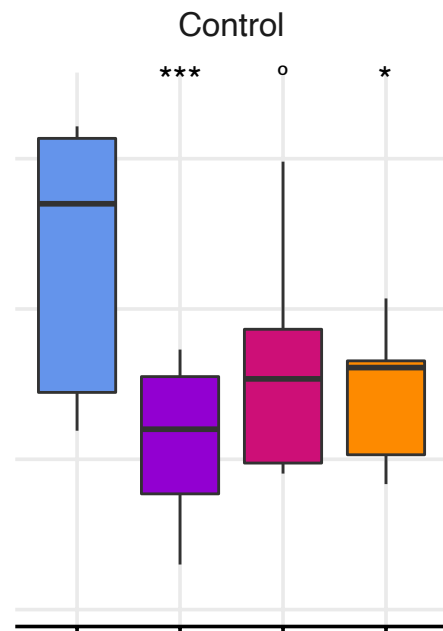
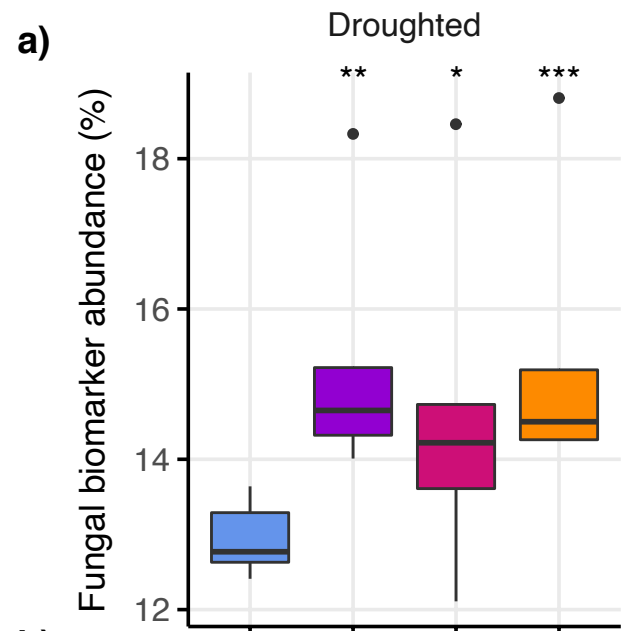
662

663 **Figure 3. Fungal biomarker functional groups following phytohormone additions to soils from**
664 **long-term climate change treatments.** Relative abundances of a) saprophytic fungi and b) arbuscular
665 mycorrhizal (AM) fungi were measured in soils collected from control, droughted and irrigated plots
666 in the Buxton Climate Change Impacts Study and incubated for 24 h with abscisic acid (ABA), 1-
667 aminocyclopropane-1-carboxylic acid (ACC), jasmonic acid (JA), or procedural controls (H₂O); boxes
668 denote the 25th and 75th percentiles and median lines are given for $n = 5$ replicates based on

669 independent field plots; whiskers indicate values up to $1.5 \times$ the interquartile range, and dots
670 indicate outliers; effects of hormone application within climate treatments are shown as *** for $p <$
671 0.001 ; ** for $p < 0.01$, * for $p < 0.05$ and ° for trends at $p < 0.1$.







1 Emma Sayer et al. use a 25-year field experiment to investigate how microbial community responses
2 to phytohormones are affected by drought. Phytohormone-induced shifts in microbial functional
3 groups suggest that drought adaptation within soil microbial communities mediates responses to
4 plant stress signalling.