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Mode of action of a novel synthetic auxin herbicide halauxifen-methyl

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Abstract

Halauxifen-methyl is a new auxin herbicide developed by Corteva Agriscience (Wilmington, DE, USA). It has been suggested that *ABF5* may be the target of halauxifen-methyl, as *AFB5* mutants of *Arabidopsis thaliana* are resistant to halauxifen-methyl, which preferentially binds to *AFB5*. However, the mode of action of halauxifen-methyl has not yet been reported. Therefore, the aim of the present study was to reveal the mode of action of halauxifen-methyl by exploring its influence on indole-3-acetic acid (IAA) homeostasis and the biosynthesis of ethylene and Abscisic Acid (ABA) in *Galium aparine*. The results showed that halauxifen-methyl could disrupt the homeostasis of IAA and stimulate the overproduction of ethylene and ABA by inducing the overexpression of 1-aminocyclopropane-1-carboxylate synthase (*ACS*) and 9-cis-epoxycarotenoid dioxygenase (*NCED*) genes involved in ethylene and ABA biosynthesis, finally leading to senescence and plant death.

KEYWORDS

halauxifen-methyl; mode of action; auxin homeostasis; ethylene; Abscisic Acid

1 INTRODUCTION

Synthetic auxin herbicides include a remarkable suite of chemical compounds that preferentially have profound morphological effects on growing dicot weeds, ultimately leading to plant death [1]. 2,4-dichlorophenoxyacetic acid (2,4-D) is among the most auxin-active molecules and was commoditized as the first auxin herbicide in the 1940s. The commercialization and popularization of 2,4-D marked the beginning of a new era of weed control in modern agriculture. Since then, various chemical classes of auxin herbicides, with different herbicidal spectra and modes of action, have been synthesized, commoditized, and become effective weed control tools[2]. Up to now, there are approximately 20 commercial compounds and even more experimental compounds that are classified as auxin herbicides, all of which contain an aryl group with an attached carboxylic acid functionality and can be divided into several categories based on their chemical structure differences [1]. Currently, synthetic auxin herbicides are classified into phenoxycarboxylic (2,4-D), benzoic (dicamba), pyridinecarboxylic (fluroxypyr), and quinolinecarboxylic (quinclorac) acids [2]. In the past few years, new auxin herbicides, such as pyrimidine carboxylic acids, aminocyclopyrachlor, and 6-aryl-picolinate herbicides, such as halauxifen-methyl (ArylexTM active) have been developed [1, add Schmitzer et al and Epp et al in here]. It is remarkable that new synthetic auxin herbicides are still being introduced today, which indicates that this unique plant-specific mode of action (MoA) is still valuable and relevant to modern agriculture.

Indole-3-acetic acid (IAA), the endogenous auxin, plays an important role in all aspects of plant development. It can promote growth and germination at low concentrations, while the opposite is true when it is used at high concentrations [3]. The effects of synthetic auxin herbicides on plants are similar to those induced by excessive treatment with the natural plant hormone auxins, such as IAA [2]. The perception and signaling pathway of endogenous auxin has long been identified [4-6]. On the contrary, although synthetic auxin herbicides have been used for more than 70 years, the precise mode of action of them has not been fully known. The perception and signaling pathway

60 of endogenous auxin is considered to trigger plant death by auxin herbicides [2, 7].
61 Through the investigation of *Arabidopsis thaliana* auxin resistant mutant lines, auxin
62 receptor and signaling pathway that essential for the plant perception and specificity of
63 auxin herbicides were discovered, revealing the ligand/receptor system [8]. The
64 *TIR1/AFB* gene family consists of six receptors: *TIR1* and five homologs of *AFB* [9-
65 11]. Auxin perception and signaling is conceptually straightforward: substrate receptor
66 binding leads to the degradation of inhibitors and the activation of transcription factors.
67 Analysis of *Arabidopsis thaliana* mutant lines has proved that auxin receptor genes are
68 involved in plant perception and specificity of auxin herbicides. For example, the *TIR1*
69 mutants are resistant to dicamba and 2,4-D [12]. In addition, the in vitro auxin receptor
70 binding studies show that auxin herbicides can bind with auxin receptors, such as
71 picloram interact preferentially with *AFB5* [13]. The *TIR1/AFB* receptors link binding
72 of auxin herbicides directly leads to the activation of transcription factors and
73 overexpression of auxin-responsive genes, which in turn cause a subsequent series of
74 biochemical and physiological events related to the action of auxin herbicides [2].
75 Amongst the auxin-response genes is the family of *ACC* synthases (ACS). Auxin
76 herbicides induce overexpression of *ACS* genes resulting in increases of ACC synthase
77 activity leading to an increase in ethylene formation. Kraft et al. [14] observed increased
78 expression of *ACS* and an increase in ethylene levels in *Galium aparine* after treatment
79 with auxin herbicides. In addition, ABA accumulation was found in a variety of dicot
80 species after treatment with auxin herbicide [15]. The key step of ABA biosynthesis is
81 the oxidative cleavage of 9-cis-epoxide carotenoid to xanthine aldehyde by 9-cis-
82 epoxycarotenoid dioxygenase (NCED), the enzyme is encoded by a family of *NCED*
83 genes [16, 17]. Kraft et al. [14] found that auxin herbicides can up-regulate gene
84 expression of *NCED* and abscisic acid accumulation in the shoot tissue of *Galium*
85 *aparine*. Excessive ethylene and ABA is thought to be the primary mode of action of
86 auxin herbicides [14, 15, 18, 19].

87 Halauxifen-methyl is one of the 6-aryl picolinates which is a new family of auxin
88 herbicides (Epp et al) developed by Corteva Agriscience. The structure of this novel
89 auxin herbicide is built on the picolinic acid scaffold and the 6-aryl group makes it

90 obviously different from the structure of all the other auxin-type herbicides listed above
91 [20]. The halauxifen-methyl can be absorbed by the leaves, translocate systemically
92 through the phloem and xylem stream, and finally accumulates in the meristematic
93 tissue [21]. When used as a herbicide, its symptoms in susceptible plants are similar to
94 those caused by other auxin herbicides, including epinasty, deformation, necrosis, and
95 eventual plant death [21]. According to the study of Dow AgroSciences, *AFB5* mutants
96 of *Arabidopsis thaliana* are resistant to halauxifen-methyl, suggesting that *AFB5* may
97 be the target of halauxifen-methyl [1, this reference is really Walsh et al, 2006]. Another
98 proof that helps confirm that halauxifen-methyl preferentially binds to *AFB5* over *TIR1*
99 is SPR binding studies [13]. However, there are few studies on the mode of action of
100 this novel herbicide post receptor. In *Brassica napus*, halauxifen-methyl treatment leads
101 to the upregulation of auxin and hormone responses, such as IAA, ABA and ACC
102 concentration [22]. McCauley et al. [23] found that halauxifen-methyl enhanced the
103 expression of NCED and led to rapid biosynthesis of ABA in *Erigeron canadensis*. In
104 the present study, the influence of halauxifen-methyl on the IAA homeostasis, ethylene
105 and ABA biosynthesis of *Galium aparine* were determined to explore the mode of
106 action of halauxifen-methyl on this common dicotyledonous weed of wheat fields [24].
107

108 **2 Materials and Methods**

109

110 **2.1 Plant materials and cultivation of plants**

111 *Galium aparine* seeds for experiments were collected in the summer of 2017 from
112 wheat fields in Minhe Village, Jiangdu Fairy Town, Yangzhou City, Jiangsu Province,
113 China. Dormancy-broken seeds were pregerminated in open trays in the illumination
114 incubator (25 °C). They were then germinated in vermiculite substrate moistened with
115 clear water in illumination incubator (day/night: 14/10 h at 25/20 °C). When seedlings
116 grow at the first whorl stage, transferred them to 1/2 Linsmaier-Skoog nutrient solution
117 and raised to the three-whorl stage in illumination incubator (day/night: 14/10 h at
118 25/20 °C). When plants grow at the three-whorl stage, transferred the uniformly
119 developed plants into 320 mL plastic cup in strength 1/2 Linsmaier-Skoog medium,

120 each cup contained 10 plants, and then put the plastic cup in illumination incubator
121 (day/night: 14/10 h at 25/20 °C). The solution was changed every three days. After a
122 week of adaptation, halauxifen-methyl (final concentration was 0.5, 5 and 50 μM
123 respectively), IAA (final concentration was 1, 0.1 and 0.01mM) was added to the
124 medium in N,N-Dimethylformamide (DMF) (0.1 % final concentration of DMF). At
125 various times (0, 6, 12, 24 h) after treatment, the shoots from parallel cups were
126 harvested, immediately frozen in solid liquid nitrogen, and then stored at -80 °C.
127

128 **2.2 Determination of ethylene production**

129 The influence of halauxifen-methyl on ethylene production of *Galium aparine* was
130 examined by following method [25]. After treatment with halauxifen-methyl in
131 hydroponic solution, the fresh weight (FW) of treated plants was measured, then were
132 transferred into 20mL head space bottle with 200μL ultrapure water in it (one plant per
133 bottles; three replications). The bottles with plants were sealed with metal caps that
134 cover with a septum. After incubation for a further 3h in the illumination incubator
135 (25 °C), a 1-mL gas sample of the head space was taken from each bottle and was
136 measured immediately by gas chromatography (GC9790Plus, Fuli Analytical
137 Instrument Co., Ltd) equipped with a flame ionization detector and a 30 m × 0.32 mm
138 × 0.25 mm Al_2O_3 column. The column temperature was 50 °C, the injector temperature
139 was 150 °C, the carrier gas flow was set to 90 ml min^{-1} and the oxidant gas flow was
140 set to 75 ml min^{-1}.
141

142 **2.3 Determination of ACC content**

143 Previous methodology [26] with slight modifications was used to measure the
144 ACC content in *Galium aparine*. 200 mg plant material was powdered in liquid nitrogen,
145 then extracted with 70% (v/v) aqueous ethanol. In order to remove the ethanol, extract
146 was centrifuged for 10 min at 10,000 rpm at 4 °C. The supernatant was passed through
147 a 0.2 μM filter. Following this, the supernatant was converted to ethylene, and then was
148 quantified by gas chromatography.
149

150 **2.4 Determination of ACC synthase activity**

151 The ACC synthase activity of treated *Galium aparine* were measured as following
152 method [27]. After being powdered under liquid nitrogen, 200 mg plant material was
153 homogenized in 2 mL 100 mmol·L^{-1} potassium phosphate buffer (pH 8.5), which
154 contain dithiothreitol (5 mM), leupeptin (10 μM) and pyridoxal phosphate (6 μM). The
155 extract was centrifuged for 10 min at 10,000 rpm at 4°C and the supernatant was passed
156 through a Sephadex G25 column. Subsequently, 0.3 mL crude extraction liquid was
157 mixed with 0.3 mL ACS assay mixture [in 80 mM potassium phosphate buffer (pH 8.5)],
158 containing 20 μM PLP and 100 μM SAM. After two hours of incubation at 37 ℃, added
159 20 μmol mercury (II) chloride to stop the reaction. And then, the ACC produced was
160 quantified by chemical conversion to ethylene. The ACC synthase activity was
161 described as ACC production rate. The background level of ACC can be measured by
162 converting them to ethylene prior to the reaction.

163

164 **2.5 Determination of ACC oxidase activity**

165 ACC oxidase was extracted and assayed as described by Dupille and Zacarías [28].
166 After weighing, individual treated plants were carefully transferred into 5 ml glass vials
167 with plastic caps, in which threre was 3 mL 5 mM ACC solution [in 25 mM potassium
168 phosphate buffer (pH 5.3)] and then sealed with a septum. After incubating at 25 ℃ in
169 darkness for 1h, a 1-mL gas sample of the head space was taken for ethylene
170 measurements by gas chromatography. The ACC oxidase activity is expressed in terms
171 of ethylene production rate.

172

173 **2.6 Determination of IAA and ABA**

174 For IAA and ABA determination, 5 g plant material was powdered in liquid
175 nitrogen and then extracted with 80% (v/v) aqueous methanol containing 1mM
176 butylated hydroxytoluene (BHT) for 12 h (three replicate extractions). To remove the
177 ethanol, extract was centrifuged for 15 min at 10,000 rpm at 4 °C. The residue was
178 extracted with 80% (v/v) aqueous methanol again. The supernatant was combined and
179 passed through a 0.2-μM filter. The volume fraction of methanol was 33% by diluting

180 the sample extract with distilled water, after which used ammonia to adjust the pH of
181 the sample extract to 8.5. Following this, the sample extract was pass through the SPE
182 column (MAX, Thermo Fisher Scientific), which had been equilibrated with 5mL
183 methanol and 5mL 2% ammonia spirit. Wash column with 5 mL 2% ammonia spirit
184 and 5mL methanol. Then applied 5 mL 1% formic acid-methanol as elute solvent to
185 MAX column and collected the efflux, which contained phytohormones of neutral and
186 acidic character: IAA and ABA. The efflux was concentrated in termovap sample
187 concentrator to dryness, and dissolved in 300 μL methanol for High Performance Liquid
188 Chromatograph (HPLC) analysis. HPLC conditions: chromatographic column: C_{18}
189 column (Agilent, 4.6×250 mm), column temperature is room temperature, run gradient
190 of A: 0.6% formic acid acid in ultrapure water, B: 100% methanol and C: 100%
191 acetonitrile, A: B: C=55: 40: 5 (v/v/v), flow rate: 1 ml/min, UV detection wavelength
192 was set at 269 nm, the injection volume is 20 μl.
193

194 **2.7 Molecular cloning of the *GaACS4*, *GaACS7* and *GaNCED1* fragment**

195 The *GaACS4* gene fragment was cloned based on homology to GenBank entries
196 for *ACS4* from *Arabidopsis thaliana* (accession NM_127846), *Solanum tuberosum*
197 (accession XM_006345517), *Solanum lycopersicum* (accession NM_001246946),
198 *Pisum sativum* (accession KX255646), *Momordica charantia* (accession FJ459814)
199 and *Ricinus communis* (accession DQ300359). Based on the conserved sequences
200 between the various cDNA clones, a pair of primers: forward ATGGGTCTTGCGGA-
201 AAATCA, and reverse GCGAAACAAACTCTAAACCA were designed. The primers
202 of *GaACS7* (forward CAGATGGGATTGGCAGAAAAT, reverse CAAAGCAAACC-
203 CTGAACCAACC) and *GaNCED1* (forward CGCAATTACTGAGAACTTCGTC,
204 reverse CGAGTTTGTTTCGGTTCACCATTC) was designed as described above
205 (Kraft et al., 2007). PCR conditions were 95 °C for 3 min and 35 cycles of 95°C for 15
206 s, 52 °C for 15 s, and 72 °C for 1 min, last 72 °C for 10 min. After DNA sequencing,
207 the resulting fragment was cloned into NCBI BLAST and confirmed for homology with
208 the *ACS4*, *ACS7* and *NCED* genes from other plants.

209

210 **2.8 Gene expression analysis based on real-time quantitative PCR**

211 Based on the DNA fragments obtained above, the primers of *GaACS4* (forward T-
212 CCAGAAATACAGCCCTGCA, reverse GACCCAAACACAGCGCTTAA), GaACS7
213 (forward CTTGACCAACCCTTCGAACC, reverse TTGCACTCAACGTCGTCTTC)
214 and *GaNCED1* (forward TGATTTCCCCGTCCTTGTGT, TGGCGAGGTTTGGAGT-
215 TTTG), for real-time quantitative PCR (qRT-PCR) was designed respectively by using
216 Primer Premier 5.0 software. Total RNA was isolated from shoot tissue of treated
217 *Galium aparine* by using RNAsimple Total RNA Kit (TIANGEN, Beijing, CHina), and
218 reverse transcribed into cDNA by using PrimerScriptTM RT reagent Kit (Vazyme,
219 Nanjing, China). The Ga28S gene that was stably expressed in many plant tissues and
220 under various stress conditions, was selected as the reference sequence(Su et al., 2020).
221 And the primers of *Ga28S* (forward TTGTCCGCATCAAAACTGGG, AACGACTAT-
222 TCCGGCACTCT) for qRT-PCR was refered to the report of Su et al.[29]. QRT-PCR
223 reactions was performed in 20-μl volume using ChamQ SYBR qPCR Master Mix
224 (Vazyme, Nanjing, China), each containing 10 μL SYBR Green Supermix, 0.4 μL 10
225 μM primers (F/R), 0.4 μL Rox, 2 μL diluted cDNA and 6.8 μL Nuclease-Free H_2O. The
226 qRT-PCR reaction conditions included one cycle of 30 s at 95 °C, 40 cycles of 10 s at
227 95 °C and 30 s at 60 °C. Relative transcript levels were calculated using the $2^{-\Delta\Delta Ct}$
228 method.

229

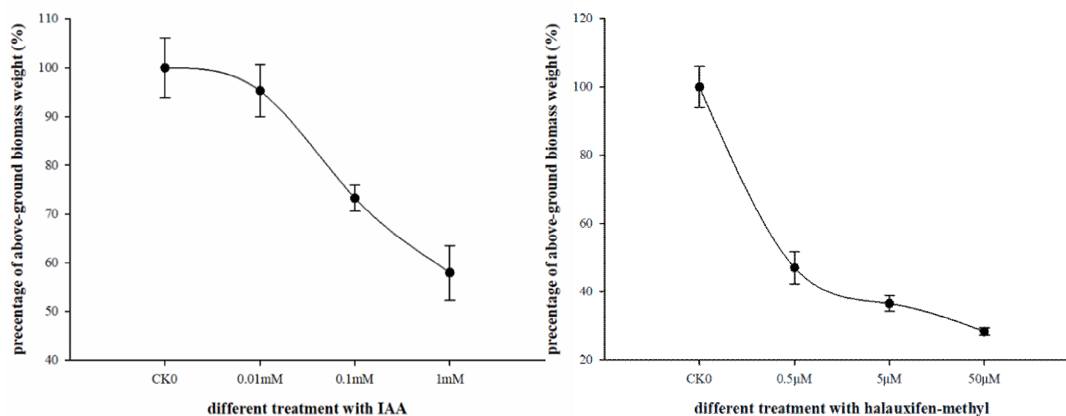
230 **3 Results**

231

232 **Effect of halauxifen-methyl on growth of *Galium aparine***

233 Within 24 h after treatment with halauxifen-methyl, some symptoms can be
234 observed, such as leaf epinasty, tissue swelling and stem twisting, which was similar to
235 those after application with excessive IAA. 5 days after application, the growth
236 inhibition was more obvious than that in 24 h and even senescence or death. At this
237 point, we measured the fresh weight of the whole plant after different treatment. The
238 results show that the fresh weight of *Galium aparine* plants was significantly reduced

239 after the use of halauxifen-methyl, and the degree of reduction was related to the
240 concentration of the herbicide (fig.1).

241 

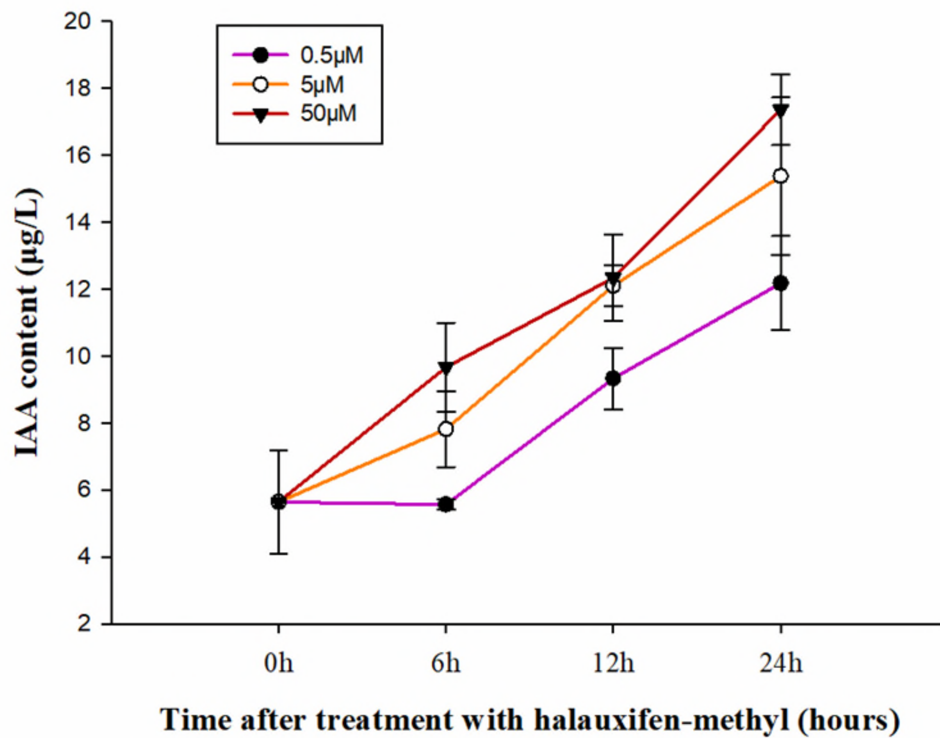
242 Fig.1 The effect of halauxifen-methyl and IAA at different concentrations on the above-ground
243 biomass of *Galium aparine*. Data are expressed as percent of the mean untreated control plants.
244 Vertical bars represent the standard errors of the means.

245

246 **Effect of halauxifen-methyl on the content of IAA in *Galium aparine***

247 *G. aparine* were treated with 0.5, 5, 50 μM halauxifen-methyl respectively by
248 applying it to the nutrient solution. IAA levels in shoot continued to increase within 24h
249 after treated and were 1.9-fold, 2.4-fold and 2.9-fold higher than those in control,
250 respectively (fig.2).

251



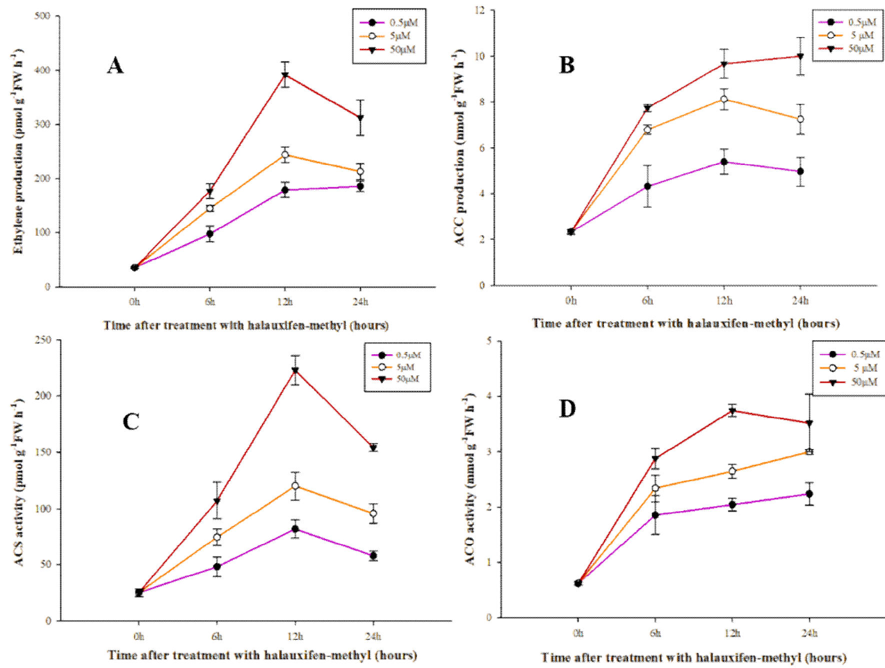
252 Fig.2. The effect of halauxifen-methyl on IAA level in shoots of *G. aparine*. Data are expressed as
253 percent of the mean untreated control plants. Vertical bars represent the standard errors of the
254 means.

255

256 **Effect of halauxifen-methyl on ethylene biosynthesis in *Galium aparine***

257 To determine the relationship between herbicidal action of halauxifen-methyl and
258 ethylene biosynthesis in *Galium aparine*, ethylene production capacity, ACC contents,
259 ACC synthase activity and ACC oxidase activity in *Galium aparine* were measured
260 after halauxifen-methyl treatment. After treated with halauxifen-methyl, ethylene
261 production of *Galium aparine* continued to increase within the first 12 h and slightly
262 decreased later. Within 24 h, the maximum ethylene production increased to 5-fold, 6.8-
263 fold and 11-fold respectively higher than those in control plants (Fig.3A). Treat with
264 halauxifen-methyl resulted in a concentration-dependent increase of ACC levels in
265 *Galium aparine* within 12 h (Fig.3B). The time course showed that maximum ACC
266 levels were reached at the 12 h and then decreased slightly. At 12 h after treatment,
267 ACC levels were 3.7-, 6.3- and 8.1- times as much as the control. The time course of

268 ACC synthase activity in treated plants was similar to ACC levels', ACC synthase
269 activity reached its peak at 12h after treatment, at which the enzyme activity increased
270 to 3.3-, 4.8- and 8.5-times as high as that of the control (fig.3C). As for ACC oxidase
271 activity, treated with 0.5 and 5μM halauxifen-methyl, it continued to increase within 24
272 h, whereas, ACC oxidase activity increased at first and then decreased after treated with
273 50 μM halauxifen-methyl. The maximum ACC oxidase activity was 3.4-fold, 4.8-fold
274 and 6.1-fold respectively higher than that of control (fig.3D). The results reveal that
275 halauxifen-methyl treatment can stimulate the activities of ACC synthase and ACC
276 oxidase, which are key rate limiting enzymes in ethylene synthesis, in a short time, thus
277 leading to the increase of ethylene precursor ACC and ethylene production.
278 To further determine the effects of halauxifen-methyl on the ethylene biosynthesis
279 pathway of *Galium aparine*, the gene expression of *GaACSs* that encode the key rate
280 limiting enzymes for ethylene biosynthesis were measured after treating by 5 μM
281 halauxifen-methyl. Studies have shown that exogenous application of IAA, ethylene
282 and ACC can increase the expression of *ACS4* and *ACS7* in Arabidopsis [30, 31], base
283 on which, we hypothesized that the synthetic auxin herbicide halauxifen-methyl, which
284 has a similar effect to IAA, can increase the expression of *ACS4* and *ACS7* genes as
285 well, therefore we selected *GaACS4* and *GaACS7* as experimental genes. The results
286 showed that expression levels of *GaACS4* and *GaACS7* continued to increase within 24
287 h of halauxifen-methyl treatment and up to a maximum of 6.8-fold and 8.1-fold
288 respectively greater than in controls at 24 h (fig.4).



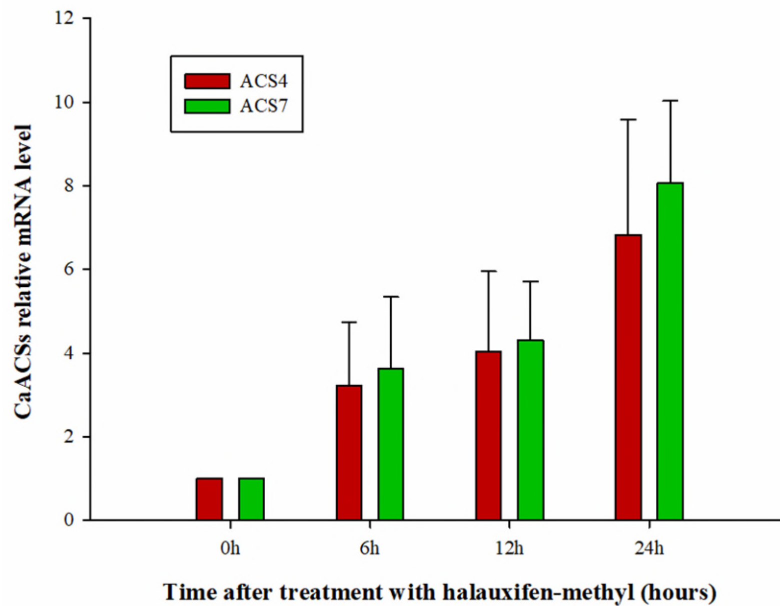
289

290 fig.3. The effect of halauxifen-methyl on ethylene production capacity (A), ACC contents (B),

291 ACC synthase activity (C) and ACC oxidase activity (D) of treated *Galium aparine*. Data are

292 expressed as percent of the mean untreated control plants. Vertical bars represent the standard

293 errors of the means.



294

295 Fig.4. The *GaACS4* and *GaACS7* gene expression patterns in *G. aparine* at 0, 6, 12 and 24 h after

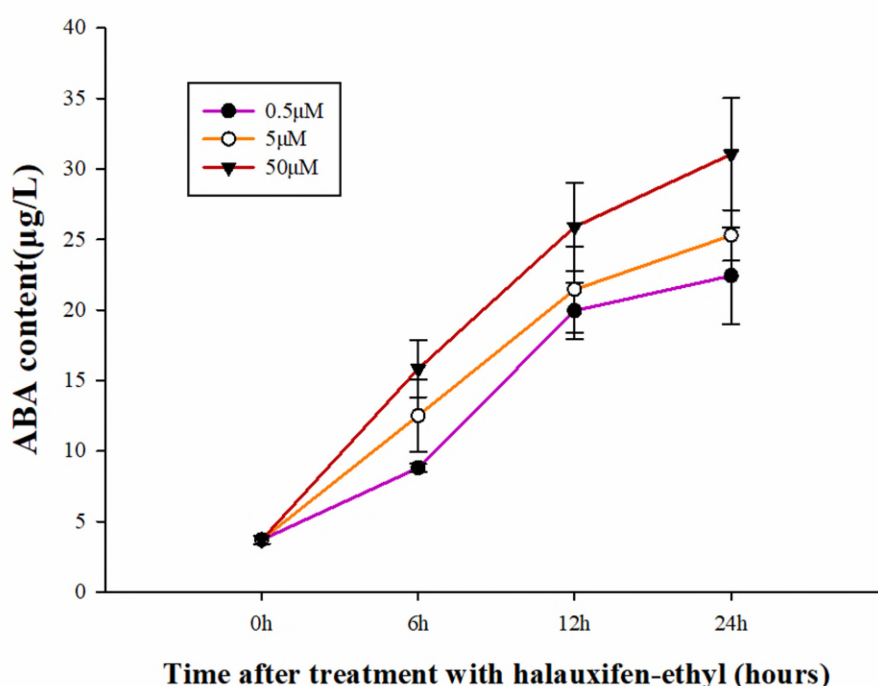
296 treated with 5 μM halauxifen-methyl. Vertical bars represent the standard errors of the means.

297

298 **Effect of halauxifen-methyl on ABA biosynthesis in *Galium aparine***

299 Abscisic acid is also one of the five plant hormones involved in plant responses to
300 abiotic stress, stomatal closure and regulation of senescence. Several authors have
301 observed that the application of auxin herbicides can lead to the accumulation of ABA
302 in plants [14, 23, 32]. In this experiment, we measured the changes of ABA content in
303 *Galium aparine* at 6, 12 and 24 h after treatment with halauxifen-methyl. The results
304 showed that the ABA contents continued to increase within 24 h after treatment with
305 halauxifen-methyl (fig.5).

306

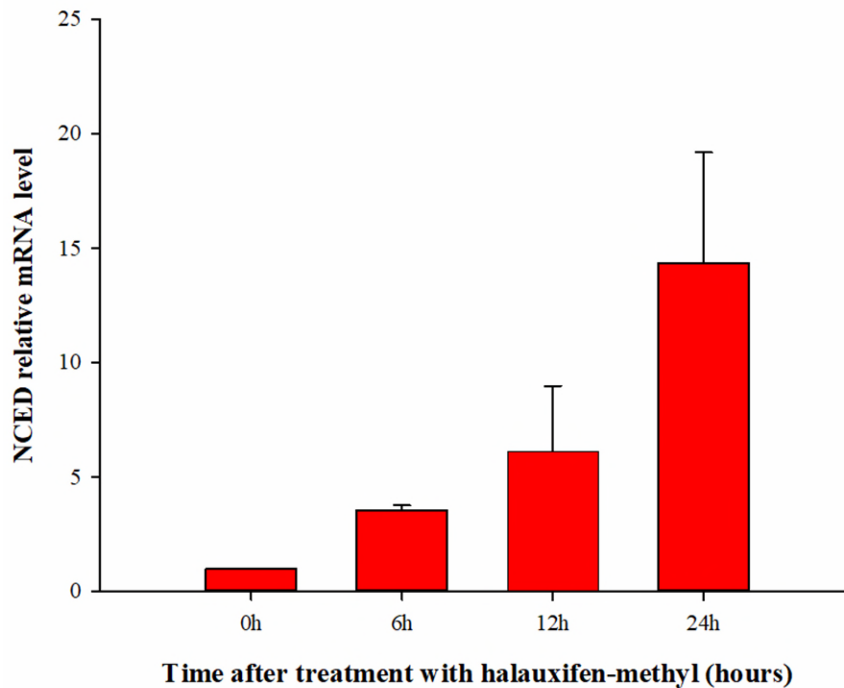


307 Fig.5. The effect of halauxifen-methyl on ABA level in shoots tissue of treated *Galium aparine*.
308 Vertical bars represent the standard errors of the means.

309

310 ABA increasing induced by auxin had been due to xanthophyll cleavage mainly
311 [33]. 9-cis-epoxycarotenoid dioxygenase (*NCED*) is the key rate-limiting enzyme of
312 ABA biosynthesis, involving in xanthophyll cleavage [34-36]. The *NCED* is encoded
313 by the *NCED* gene family [37, 38], and after treating with 5 μM halauxifen-methyl, the
314 *GaNCED1* gene in *Galium aparine* was consistently upregulated within 24 h (fig.6).
315 The herbicide treatment at 24 h resulted in the highest gene expression level of
316 *GaNCED1*, which was 14.4-fold higher than those in controls.

317



318 Fig.6. The *GaNCED1* gene expression patterns in t *Galium aparine* at 0, 6, 12 and 24 h after

319 treated with 5 μM halauxifen-methyl. Vertical bars represent the standard errors of the means.

320

321 ## 4 Discussion

322 Synthetic auxin herbicides (SAHs) are one of the most widely used kinds of herbicide

323 worldwide [2]. Halauxifen-methyl is a new SAH representing a new chemical class

324 [22], and it has been widely used in China for the weed control in paddy rice fields,

325 especially for the *Echinochloa* species.

326 In general, the mode of action of SAHs involves over-induction of auxin responses

327 in susceptible plants, such as production of ethylene and ABA. It is well reported that

328 upregulation of ethylene biosynthesis accounts for a large part of the repertoire of

329 SAHs-mediated responses, and it's a typical early reaction in various weeds such as

330 *Echinochloa crusgalli* var. *zelayensis* with quinclorac, *Galium aparine* with dicamba

331 and with picloram [2, 4, 39-41]. Following ethylene overproduction, ABA accumulates

332 in plants [25, 39, 41] and several studies have shown that ABA plays a decisive role in

333 the action of many SAHs [14, 23, 25, 36, 42, 43]. In the present study, overproduction

334 of ethylene and the accumulation of ABA were observed in *G. aparine* when treated

335 with halauxifen-methyl, suggesting that halauxifen-methyl shares the same set of
336 phytotoxic responses and extending range of weed families shown to respond to these
337 new herbicides.

338 Application of SAHs lead to auxin overdose which, like excess endogenous auxin
339 concentrations is likely to lead to the imbalance of auxin homeostasis [2]. Studies have
340 shown that 2,4-D treatment, for example, may cause either a decrease or an increase
341 level of free IAA in plants [45]. In the case of G. aparine treated with halauxifen-methyl,
342 it was shown that endogenous IAA levels rise within 6 hours and continue to rise for
343 24 hours (Fig. 2). At the lowest dose of halauxifen-methyl, the rise in endogenous IAA
344 appears slower, but in all cases at all doses the plants are overwhelmed by the additional
345 load of the synthetic auxin in the treatment. The combined rise in auxins induced
346 dramatic rises in ethylene and ABA concentrations in *G. aparine* (Fig 3A and Fig 5).
347 The ethylene concentration rose more than three-fold within 6 hours, and between five-
348 and ten-fold within 12 hours, with these high levels maintained for at least 24 hours.
349 The rising levels of ethylene correlated with rises in expression of the genes encoding
350 ACS4 and ACS7 (Fig 4), in the activities of the biosynthetic enzymes ACS and ACO
351 (Fig 3C and 3D), and in the accumulation of the intermediate ACC (Fig 3B).

352 The rise in ABA concentration in treated *G. aparine* (Fig. 5) was as rapid and as
353 extreme as for ethylene. Within 6 hours ABA levels had risen by at least three-fold and
354 by five- to ten-fold after 24 hours. These rises in ABA correlated well with elevations
355 of expression of the gene coding for the rate-limiting enzyme in biosynthesis, NCED.
356 (Fig 6). Taken together, all the data support the hypothesis that the mode of action of
357 halauxifen-methyl in *G. aparine* is as an SAH, with herbicidal doses leading to rapid
358 and extreme elevations in the expression of genes which code for enzymes that
359 biosynthesize ethylene and ABA[44]. The exogenous SAH also led to rises in
360 endogenous IAA accumulation and this may have contributed to the set of acute
361 responses downstream from auxin perception.

362

363 The treatment of *G. aparine* with halauxifen-methyl has shown that this new
364 herbicide follows the paradigm of SAH activity on dicot weeds. However, it is worth

365 noting that the induction of ACC synthase or ACC oxidase expression has not been
366 observed in all cases and might be dose-dependent. In *Arabidopsis,* Raghavan et al.
367 (2006) found that the expression of *ACS* and *ACO* was up-regulated after treatment with
368 0.001 and 0.01 mM 2,4-D, but was not changed after treatment with 0.1 and 1mM 2,4-
369 D [32]. The expression of the *CTR1* gene, a negative regulator of ethylene signaling
370 was down-regulated correspondingly [32, 51], which suggested that there was more
371 than one pathway for ethylene to respond to auxin herbicides. McCauley et al. [23]
372 studied the rapid responses to SAHs in *Erigeron canadensis* using transcriptomics and
373 targeted physiological studies, and found that though ABA accumulation was observed,
374 there was no significant difference in the expression of ethylene biosynthesis genes.
375 therefore, in some plants ethylene synthesis may not be necessary to trigger ABA
376 accumulation.
377 In some plants, notably *E. crusgalli* var. *zelayensis,* resistance to applied SAHs has
378 been conferred by reduced ethylene biosynthesis or reduced sensitivity to ethylene
379 (REFS – your papers) which emphasizes the importance of ethylene in the herbicidal
380 responses of some plants. Other sources of resistance to SAHs have also been reported
381 including both target site and non-target site resistances (Busi et al., Rey-Caballero et
382 al).
383
384 In the present study, application of halauxifen-methyl resulted in an increase in
385 IAA content, which contributed to the overexpression of *ACS* and *NCED* genes in *G.*
386 *aparine.* These transcripts helped elevate the activity of key rate-limiting enzymes in
387 ethylene and ABA biosynthesis pathways respectively, which led to the overproduction
388 of ethylene and ABA and, eventually plant death. Given the specificity of halauxifen-
389 methyl for AFB5 in the auxin receptor family (Walsh et al, and 13), care must be taken
390 to monitor for weed resistance to this useful new SAH and further studies on its
391 mechanism of action are needed.
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