1 **Title:** Flooding increases respiration and sugar content in the tomato stem: survival

2 strategy or "aimless" response?

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12

17 **Abstract**

18 With flooding being one of the numerous challenges that ecosystems face throughout

19 the world, plants are therefore obliged to adopt plastic responses in order to cope with

20 this environmental constraint. When flooded, the tomato hypocotyl undergoes profound

21 changes that entail rearrangements in its physiology and metabolism. In this work, we

22 observed that, although soil flooding markedly dampens root respiration, the submerged

23 hypocotyl surprisingly enhances oxygen consumption in spite of hypoxic conditions.

24 Several pieces of evidence indicate that the respiratory pathway is indeed promoted in

25 submerged stems. Besides, underwater hypocotyls are shown to accumulate sugars.

26   Girdling and feeding experiments revealed that leaf-derived sucrose is metabolized and

27   channelled to maintain respiration in underwater hypocotyls. Our data suggest that high

28   respiration is required for sucrose unloading from phloem, since inhibition of

29   hypocotyls respiration significantly prevents sugar build-up. As substrate availability

30   increases, respiration is fuelled even more, leading to a sustained allocation of sugars to

31   flooded hypocotyls.

32

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36   **Conflicts of Interest**

37   The authors declare no conflicts of interest.

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39   **Main Text File**

40

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58   flooded hypocotyls.

59

60   **Keywords**

61   Tomato, Flooding stress, Root hypoxia, Respiration, Soluble sugars, Sink establishment,

62   Hypocotyl, Oxygen consumption, Mitochondrial activity, Sucrose

63

64   **Introduction**

65   Flooding events have risen dramatically since the 50's, causing damage to natural

66   vegetation and crops (Pedersen et al., 2017). Considering that between 2006 and 2016

67   almost two-thirds of all damage and loss of crops was caused by floods (FAO, 2018),

68   flooding resilient crop selection represents a major challenge for scientists (Mustroph,

69   2018).

70   As obligate aerobic organisms, plants necessarily require oxygen for respiration.

71   However, in water-saturated soil and stagnant waters, oxygen entry into cells is

72   dramatically hindered due to its modest solubility and diffusion in water (Jackson and

73   Ram, 2003). In this situation, some plants plastically respond in order to cope with

74   oxygen shortage. Among morpho-anatomical adaptations, lenticel hypertrophy,

75   impermeable barriers to limit root radial oxygen loss, adventitious roots and

76   aerenchyma represent the most common ones even in non-wetland plant species

77   (Colmer, 2003; Kozlowski, 1984).

78   In oxygen deprived environments, cells rearrange their metabolism in order to limit

79   oxygen consumption and maximize ATP production. This generally implies the

80   exacerbation of glycolytic flux, the down-regulation of many components of Krebs

81   cycle and the reduction of mitochondrial electron transport activity (Bailey-Serres and

82   Voesenek, 2008; Rocha et al., 2010; Shingaki-Wells et al., 2014). Glycolysis and

83   fermentation represent the only routes that provide energy to cells under hypoxia.

84   However, due to the inefficient ATP production (only 2-4 moles ATP per mole of

85   hexose compared to 30-36 moles ATP produced by aerobic respiration), an energy crisis

86   often occurs (Pucciariello and Perata, 2012).

87   Sugars are involved in different important physiological processes throughout the plant

88   life cycle, such as respiration, photosynthesis, flowering and senescence, among others.

89   In addition, their participation as osmoprotectors, antioxidants and signalling molecules

90   allows plants to modulate their response to abiotic stress (Gangola and Ramadoss,

91   2018). Along with playing an important role as an energy source under anoxia and

92   submersion (Kudahettige et al., 2011; Loreti et al., 2005), sugars such as sucrose, have

93   been reported to induce morphological changes in submerged tissues (Qi et al., 2020;

94   Takahashi et al., 2018). Flooded plants are often characterized by an increased level of

95   sugars in roots, leaves and phloem sap. Since the photosynthetic rate usually diminishes

96   under flooding (Else et al., 2009; De Pedro et al., 2020), some of the possible

97   explanations of this increase in sugar content are an impaired sugar transport

98   mechanism and/or a reduced sugar usage in sink organs (i.e. roots) (Saglio, 1985;

99   Albrecht et al., 2004; Peuke et al., 2015). Sugar availability and its utilization in

100   hypoxic tissues have been shown to ensure cell viability and survival for they are

channelled into fermentation pathways (Cho et al., 2021; Kudahettige et al., 2011;

Loreti et al., 2005; 2018; Webb and Armstrong, 1983). Sugar consumption under

flooding involves the transcription of subsets of genes. Among them, the hypoxia

regulated genes (HGSs), which encompass genes encoding fermentation enzymes, are

activated following the hypoxia-mediated stabilization of the protein RAP2.12, an

Ethylene Responsive Factor (ERF-VII) (Licausi et al., 2011). However, prolonged

hypoxia progressively leads to starch reserves exhaustion and to energy deprivation. As

a consequence, a reduced amount of phosphate sugars such as Trehalose-6-phosphate

(Tre6P) is reported to induce the release of the cell energy sensor SnRK1 (Sucrose-non-

fermenting1-related kinase1) protein activity (Gazzarrini and Tsai, 2014). De-regulation

of SnRK1, in turn, triggers the repression of energy-consuming anabolic processes and

the activation of starvation regulated genes (Baena-González and Hanson, 2017; Cho et

al., 2021).

Tomato (*Solanum lycopersicum* L.) is a worldwide cultivated horticultural crop that in

2018 attained a global production of more than 182 M tonnes (www.faostat.org).

However, production can be harmed by soil flooding because of the marked sensitivity

of tomato roots to hypoxia (Ezin et al., 2010; Horchani et al., 2008). Indeed, prolonged

stress conditions result in an almost complete loss of root functionality and integrity

(Horchani et al., 2008; 2009). Tomato reacts to flooding injury by profoundly

reprogramming root transcriptome (Safavi-Rizi et al., 2020). Energy metabolism is also

rearranged in submerged tomato roots, since sucrose and hexoses uptake increases, and

glycolytic and fermentative pathways become predominant (Germain et al., 1997;

Gharbi et al., 2009). In addition, tomato plants respond to partial submergence with a

series of morpho-anatomical changes that involve the hypocotyl. Among them,

hypertrophy of cortex cells and lysigenous aerenchyma formation are the most evident

126 (Kawase and Witmoyer, 1980; Mignolli et al., 2020). In addition, the hypocotyl

127 represents the site where a new adventitious root system is originated (McNamara and

128 Mitchell, 1990; Vidoz et al., 2010). While aerenchyma allows oxygen to reach

129 submerged hypocotyl tissues, adventitious roots are able to restore hydraulic

130 conductivity and stomatal aperture, leading to improved photosynthetic efficiency and

131 biomass accumulation under flooding (Else et al., 2009; Vidoz et al., 2016). All these

132 plastic morpho-anatomical acclimation responses have been suggested to allow plants to

133 tolerate a few days of partial submersion and seem to have a major role in tomato plant

134 stress resilience. However, little is known about the physiological and biochemical

135 events that underlie these changes. In this paper, we provide evidence about the effect of

136 flooding on increased respiration enabling, in this way, sugar unloading and

137 accumulation in the stem. Whether or not this apparently unobvious phenomenon is part

138 of the plant's mechanisms to cope with flooding is discussed.

139

140 **Materials and methods**

141

142 *Plant material and growth conditions*

143 Seeds of tomato (*Solanum lycopersicum* L.) of the cultivar Ailsa Craig (AC) accession

144 n° LA2838A were obtained from the Tomato Genetic Resource Center (TGRC,

145 University of Davis). Seedlings were grown in 300 ml containers with peat-based

146 substrate (Growmix Multipro, Tierrafertil, Argentina), placed in a growth chamber at 26

147 $\pm$ 2 °C and 50-70% relative humidity, illuminated by high-pressure sodium lamps with a

148 photoperiod of 15 h : 9 h, light : dark and an intensity of 500 μmol m^{-2} s^{-1}, and regularly

149 watered with ¼ Hoagland solution. When seedlings were 4 weeks old, flooding

150 treatments were set up as described by De Pedro et al. (2020). For the experiment with

151 waterlogged plants, flood water reached the base of the stem so that only roots were

152 submerged.

153

154 *Oxygen measurements*

155 Oxygen consumption was measured in excised roots and hypocotyls (about 0.3 g) from

156 control and flooded plants. Samples were placed in 4 ml HPLC sealed vials. Oxygen

157 concentrations were registered immediately after closing the vials and after 2 h of

158 incubation at 37°C in dark with the oxygen microsensor OXR50 connected to the

159 FirestingO2 oxygen meter (PyroScience GmbH). An empty vial was also incubated and

160 used for temperature compensation of oxygen measurements with the external

161 temperature sensor TDIP15 (PyroScience GmbH). Oxygen uptake was expressed as ml

162 O_2 consumed g^{-1} FW h^{-1} and calculated as follows:

163
$$\frac{\left(T\frac{0*V}{100}\right)-\left(\frac{Tf*V}{100}\right)}{h*FW}$$

164 Where:

165 T0 = Initial O_2 %

166 Tf = Final O_2 %

167 V = Vial headspace volume (ml)

168 h = Incubation time (h)

169 FW = Hypocotyl/root fresh weight (g)

170

171 *Metabolite extraction*

172 Metabolites were extracted according to Tobias et al. (1992) with minor changes.

173 Frozen vegetal material, from 0.1 to 0.3 g of fresh weight, were ground in 1 ml 5.5%

174 $HClO_4$ and incubated 1 h at 4°C. Samples were then centrifuged at 14000 rpm at room

175 temperature and the supernatant was recovered. The pellet was extracted again with 0.5

176 ml 5.5% HClO$_4$ and supernatant was recovered and combined with the initial one.

177 Extracts were neutralized with 3.5 M K$_2$CO$_3$ and left to incubate for 30 min at 4°C.

178 Samples were centrifuged at 14000 rpm for 10 min to remove potassium perchlorate.

179 Supernatant was recovered and stored at -20°C up to analysis.

180

181 *Sugars determination*

182 Total water soluble sugars were determined with the phenol-sulphuric method according

183 to Buysse and Merckx (1993). Five hundred µl of sample extracts were mixed with 28%

184 phenol dissolved in 80% ethanol and 2.5 ml of concentrated sulphuric acid (98%) were

185 added. Developed colour was quantified spectrophotometrically at 490 nm against a

186 sucrose standard concentration curve.

187 Starch determination was carried out using the remaining pellet. The pellet was

188 extracted 3 times with 80% ethanol at 80 °C for 15 min each time. After centrifugation

189 for 10 min at 14000 rpm and 25°C, supernatant was discarded, the pellet was

190 resuspended with 700 µl 0.1 M NaOH and incubated at 100 °C for 1 h. After cooling,

191 samples were centrifuged and supernatant was neutralized with 1 M HCl. Subsequently,

192 50 µl of neutralized extracts were added to 0.1 U µl^{-1} of amyloglucosidase from

193 *Aspergillus niger* (Sigma-Aldrich, St Louis, MO, USA) in 200 mM acetate buffer (pH

194 4.5) and incubated for 12 h at 42 °C. Free glucose obtained after starch hydrolysis was

195 determined with the phenol/sulphuric method previously described.

196 Determination of sucrose, glucose and fructose content in control and flooded

197 hypocotyls was performed enzymatically following the method described by

198 Guglielminetti et al. (1995).

199

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200 *ATP content estimation*

201 Neutralized extracts from control and submerged hypocotyls were used to determine

202 ATP content according to the spectrophotometric procedure described by Tornheim and

203 Schulz (1990).

204

205 *Hexokinase activity*

206 Frozen hypocotyl segments of about 200 mg were ground in a pre-chilled mortar with

207 700 µl of extraction buffer. The extraction buffer consisted of 100 mM HEPES/KOH

208 (pH 7.4), 5 mM $MgCl_2$ $6H_2O$, 1 mM EDTA-NA$_2$, 1% insoluble PVPP, 10% glycerol,

209 0.1% Triton X and 5 mM DTT. Extracts were centrifuged at 14000 rpm for 15 min.

210 Crude extract was used for the glucokinase (GK) and fructokinase (FK) enzymatic

211 activity assay that was performed following the procedure described by Tomlinson et al.

212 (2004).

213

214 *COX and AOX capacity assay*

215 To inhibit cytochrome *c* (COX) pathway, excised hypocotyls (approx. 0.4 g) from

216 control and flooded plants were incubated in flasks with 50 ml of 2.5 mM KCN (Merck,

217 Germany) in 20 mM phosphate buffer pH 7.0. To block COX and alternative oxidase

218 (AOX) pathways, segments were immersed in 2.5 mM KCN + 10 mM SHAM

219 (salicylhydroxamic acid, Santa Cruz Biotechnology, USA) in 20 mM phosphate buffer

220 pH 7.0. As control, segments were incubated in 20 mM phosphate buffer pH 7.0.

221 Incubation was carried out at 27°C for 4 h under continuous shaking (100 rpm).

222 Segments were rinsed with distilled water and individually placed in 4 ml septum

223 capped vials. Oxygen uptake was determined as previously described. COX and AOX

224 capacity were calculated according to the equation: $Vt = Vcyt + Valt + Vres$ (Møller et al.,

17
18

225   1988). COX capacity (V_{cyt}) was obtained as the difference between oxygen consumption

226   of hypocotyls incubated in absence of inhibitors (V_t) and in presence of KCN (V_{alt} +

227   V_{res}). AOX capacity (V_{alt}) was determined as the difference between oxygen

228   consumption in presence of KCN and in presence of KCN + SHAM (V_{res}).

229

230   *TTC reduction assay*

231   2,3,5-triphenyltetrazolium chloride (TTC) assay was performed according to Yamauchi

232   et al. (2014) with some modifications. Hypocotyls from control and flooded plants were

233   cut in slices 0.5-1 mm thick. Slices were incubated in 0.1 M phosphate buffer pH 7.0

234   with 0.6% 2,3,5-Triphenyltetrazolium chloride (Cicarelli Laboratorios, Argentina) for 1

235   h at 37°C in the dark. Water-insoluble red formazan was extracted by keeping the slices

236   in 1 ml of 95% ethanol overnight. After complete discoloration, slices were removed

237   and ethanolic extracts were brought to 2 ml volume with 95% ethanol. Absorbance of

238   red formazan at 485 nm was measured. Reduction of TTC was expressed as $O.D._{485}$ g^{-1}

239   FW.

240

241   *Girdling experiments*

242   Phloem flow was interrupted through heat girdling according to what was described by

243   Takahashi et al. (2018). Four-week-old plants were girdled one day before the

244   beginning of the experiment. A scalpel blade was heated with the flame of a Bunsen

245   burner and, with the blunt side, a 1 mm deep annular mark was practised around the

246   stem above the cotyledonary node (high girdling, HG). Low girding (LG) was

247   performed at the neck of the plant at about 0.5 cm above soil surface.

248

249   *Feeding experiments*

250   Hypocotyl segments of about 1 cm long and 0.4 g of weight were excised from 4-week-

251   old plants. Segments were placed in flasks with 20 ml of an equimolar concentration (20

252   mM) of sucrose, glucose, fructose, maltose and turanose. Sucrose, fructose, glucose and

253   maltose were purchased from Sigma-Aldrich, whereas turanose was purchased from

254   Santa Cruz Biotechnology. Feeding solutions were prepared in 20 mM phosphate buffer

255   pH 7.0. Control solution only contained 20 mM phosphate buffer pH 7.0. Sections were

256   mildly vacuum-infiltrated with the solutions for 3 min and then placed in flasks with the

257   fresh feeding solutions. Flasks were incubated in light at 27 °C for 20 h under constant

258   agitation at 100 rpm. After incubation, segments were rinsed with distilled water,

259   weighted and placed in 4 ml septum capped vials for oxygen consumption

260   measurements as described before.

261

262   *Gene expression analysis*

263   Total RNA from hypocotyls was extracted following the procedure described by

264   Mignolli et al. (2020). Expression of *LIN6* and *SUS1* genes was performed by Real

265   Time PCR according to Mignolli et al. (2020). The expression of *LeEF1α* gene was

266   used as internal reference. Primers used for Real Time PCR reactions were: *LeEF1α*

267   (X53043) primers Fw 5′-CATCAGACAAACCCCTCCGT-3′ and Rv 5′-

268   GGGGATTTTGTCAGGGTTGTAA-3′; *LIN6* (AF506005.1) primers Fw 5′-

269   TTCGTAAGTGGATCAAGCCC-3′ and Rv 5′-GATTCCTCACACTCCCAACC-3′;

270   *SUS1* (L19762.1) primers Fw 5′-GGTTATCCTTTCCCCTCATGG-3′ and Rv 5′-

271   AGTCCTTGCTCCTTTATGCG-3′.

272

273   *Statistical analysis*

274 Normal distribution of each dataset was checked according to D'Agostino and Pearson

275 omnibus normality test, using GraphPad Prism 6.0. When normality requisite was

276 fulfilled, one-way ANOVA and Tukey's HSD Post Hoc test were performed.

277 Otherwise, the non-parametric ANOVA Kruskal-Wallis test was carried out. In both

278 cases, Infostat software 2012 version (Di Rienzo et al., 2012) was used.

279 Unpaired t test was applied when two groups of data were analysed, provided data

280 population passed normality test. Otherwise, log transformation of the values was

281 performed to obtain Gaussian distributions using GraphPad Prism 6.0.

282

283 **Results**

284

285 *Flooded hypocotyls show enhanced respiration*

286 The hypoxic condition of flooded tomato plants was checked by measuring dissolved

287 oxygen in soil (5 cm below soil surface) and in water. Dissolved oxygen in the substrate

288 sloped from 6.5 to 2.4 mg l^{-1} after 3 days from the start of the experiment. No further

289 decrease was recorded after 6 days of flooding (Fig. S1 left histogram). Dissolved

290 oxygen level in water was slightly higher than in soil and, apart from small fluctuations,

291 it was maintained around 7 mg l^{-1} (Fig. S1 right histogram).

292 Oxygen consumption rate in flooded roots almost halved after 1 day from the onset of

293 stress but no further decrease was recorded the following days (Fig. 1A left histogram).

294 In parallel to reduced oxygen consumption, root biomass in flooded plants ceased to

295 accumulate and was 2 and 3 times lower than control plants after 3 and 6 days (Fig. 1A

296 right histogram). Regardless of the low oxygen tension in water, oxygen consumption

297 rate of submerged hypocotyls was always higher than in air-exposed hypocotyls of

298 control plants (Fig 1B left histogram). We discarded the possibility of an artefact

23

24

299 resulting from the sudden reoxygenation of hypocotyls during measurements, by

300 assessing oxygen uptake of samples incubated in water-filled vials. Even in this case,

301 flooded hypocotyls showed higher oxygen consumption rate that control ones (data not

302 shown). We also tested the possibility that the increased oxygen consumption was due

303 to endophytic bacteria adhering to cells or associated with vascular bundles (White et

304 al., 2019). To this aim, we incubated hypocotyl segments from control and partially

305 submerged plants in presence of streptomycin sulphate. Oxygen consumption, however,

306 indicated that the antibiotic did not have a significant effect on control or submerged

307 hypocotyls (Fig. S2). In addition, we ascertained whether the only submersion of roots

308 and not the stem was sufficient to induce hypocotyl respiration. Therefore, we measured

309 oxygen consumption in hypocotyls of waterlogged plants, in which just roots were

310 underwater. As shown in Fig. 1B (right histogram), after 3 and 6 days, oxygen uptake

311 was twice as high in hypocotyls of waterlogged plants as in control ones.

312 In order to establish whether the enhanced oxygen consumption in submerged

313 hypocotyls was indeed associated with respiration and mitochondrial activity, we

314 proceeded to assess the respiratory metabolism by measuring the activity of GK and FK.

315 While no difference in activity for FK was observed, GK activity in submerged

316 hypocotyls was 2.5 times higher than in controls (Fig. 2A). Mitochondrial

317 dehydrogenase activity was tested with triphenyl tetrazolium chloride (TTC) reduction

318 assay. Indeed, reduction of TTC to formazan has been shown to occur mainly by the

319 action of mitochondrial Complex I (Rich et al., 2001). Red formazan was more evident

320 in sections of submerged hypocotyls, especially around vascular tissue (Fig. 2B). To

321 confirm the observation, spectrophotometric quantification of the compound revealed

322 that, since the first day of submergence, levels of formazan in underwater stems were

323 always more than double than in control plants (Fig. 2C). High oxygen consumption

25
26

324 suggests that the activity of cytochrome *c* (COX) and/or alternative (AOX) oxidase are

325 enhanced in submerged hypocotyls. To assess this, we measured COX and AOX total

326 capacity. Data showed that, in flooded hypocotyls, COX capacity was 44% higher than

327 in controls. AOX capacity was ten times lower than COX in flooded tissues, while it

328 remained undetectable in control plants (Fig. 2D). We went further to investigate

329 whether this increased respiratory activity translated into a higher production of ATP.

330 Interestingly, content of ATP in hypocotyls of flooded plants reached values that were

331 82% and 40% higher than in controls after 3 and 6 days of submergence (Fig. 2E).

332

333 *Phloem-derived sugars fuel respiration in submerged hypocotyls*

334 In order to ascertain whether leaf-derived photoassimilates sustain underwater

335 respiration in hypocotyls, we performed girdling experiments to block phloem sap

336 supply to submerged tissues. Girdling was carried out above the cotyledonary node

337 (high girdling, HG). After 3 days, no visible changes were observed in control plants

338 that only displayed adventitious root primordia (as small bumps) above the girdling

339 scar. Conversely, submerged plants showed few short adventitious roots emerging from

340 the hypocotyl below the girdling point (Fig. 3A; S3A) and lower level of porosity (Fig.

341 S3B). Significantly, girdling reduced the oxygen consumption rate in submerged

342 hypocotyls by 24% in comparison to non-girdled plants (Fig. 3B left histogram).

343 Consistently, we also observed a reduction by 38% of formazan content in submerged

344 hypocotyls, indicating a diminished mitochondrial activity (Fig. 3B right histogram).

345 We hypothesized that sucrose was responsible for fuelling respiration in flooded tomato

346 hypocotyls. To prove that, we measured the oxygen uptake of excised hypocotyls from

347 control plants after having fed them with equimolar concentrations of sucrose, glucose,

348 fructose, maltose and turanose. Interestingly, among all tested sugars, sucrose-fed

349 segments showed the highest oxygen consumption rate (Fig. 4A). When another

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28

350   disaccharide such as maltose was used, oxygen uptake was lower than in sucrose-fed

351   hypocotyls, while no effect was observed in presence of turanose (a non-metabolizable

352   sucrose analogue that activates sucrose signalling, Loreti et al., 2001). Hexoses such as

353   fructose and glucose also increased oxygen consumption, though to a lesser extent than

354   sucrose (Fig. 4A). We then asked whether the respiration rate of hypocotyls correlated

355   with the availability of sucrose. For this purpose, we fed hypocotyl segments from

356   control plants with increasing concentrations of sucrose and oxygen uptake rate was

357   measured. Not surprisingly, oxygen consumption increased linearly with the increase of

358   sucrose molarity in the incubation medium (Fig. 4B).

359   In order to investigate whether sucrose metabolism in submerged hypocotyls takes

360   place, we monitored the expression of the apoplastic invertase *LIN6* and the sucrose

361   synthase *SUS1* genes. Both genes have been shown to be chiefly expressed in stem

362   vascular tissues (Proels and Roitsch, 2009; Goren et al., 2011). Data showed that both

363   genes were strongly upregulated in submerged hypocotyls from the first day since the

364   onset of flooding (Fig. 4C, D). In particular, the expression of *SUS1* peaked after 3 days

365   reaching a level more than 40 times higher than in controls (Fig. 4D). Lower induction

366   was observed for the *LIN6* gene, being roughly 4 and 12 times higher in submerged

367   tissues at the third and sixth days, respectively (Fig. 4C).

368

369   *Respiration sustains sugar accumulation in flooded hypocotyls*

370   The amount of water soluble sugars significantly augmented in flooded hypocotyls.

371   Both in partially submerged and waterlogged plants, hypocotyl sugar levels were higher

372   than in control stems after 3 and 6 days from the beginning of the flooding treatment

373   (Fig. 5A). Analysis of sucrose, glucose and fructose content revealed that both sucrose

374   and hexoses were more concentrated in submerged hypocotyls (Fig. 5B). Sucrose was

the most abundant and reached levels that were 3 and 2.5 times higher than in controls

at the third and sixth days, respectively. Similarly, both glucose and fructose content

also increased and was, respectively, 4- and 3-times higher than in control hypocotyls.

Starch was also measured and its content was higher than in non-flooded plants after 6

days from the start of the experiment (Fig. 5C).

Furthermore, we explored whether sugar accumulation in flooded hypocotyls depended

on leaf-derived phloem sap. To this aim, we analysed the sugars content in intact and

girdled plants under flooding conditions. When the phloem flow was blocked by

girdling above cotyledons (HG), sugars level was about 2.5-fold lower than in intact

plants (Fig. 5D). Following, we sought to assess if the disruption of the root sink in

flooded plants determines the increase in respiration and sugar content in hypocotyls.

To do so, we girdled non-flooded plants at the base and measured oxygen uptake rate

and soluble sugars content in hypocotyls, and compared these values with those from

waterlogged plants. Notably, while waterlogged plants showed high oxygen

consumption rate and sugar levels, girdling in non-flooded plants did not cause any

significant change neither in oxygen uptake nor in sugar content in the hypocotyl if

compared with intact plants (Fig. 5E).

Finally, we asked whether respiration in flooded hypocotyls is required to sustain sugars

accumulation. To address this question, we partially submerged plants in a solution of

0.5 mM KCN in order to block hypocotyl respiration. After 3 days, oxygen

consumption and soluble sugars content was measured in hypocotyls submerged in

water with or without KCN. Interestingly, along with a significant halt in oxygen

uptake, KCN-submerged hypocotyls showed lower sugar accumulation by nearly 3-fold

(Fig. 5F).

Discussion

As widely reported, organs or tissues exposed to hypoxia, adapt their metabolism in order to save oxygen (Bailey-Serres and Voesenek, 2008; Cho et al., 2021; Geigenberger et al., 2003; Rocha et al., 2010). In our experimental conditions, roots and hypocotyls of partially submerged tomato plants were exposed to oxygen concentrations well below the atmospheric one (Fig. S1). Flooding caused growth arrest and progressive root decay due to oxygen shortage (Fig. 1A, McNamara and Mitchell, 1989; Vidoz et al., 2016). Surprisingly, submerged hypocotyls show an enhancement of oxygen uptake (Fig. 1B left histogram). Interestingly, elevated oxygen consumption was observed in hypocotyls of waterlogged plants, where only roots were underwater (Fig. 1B right histogram) suggesting that the increase of oxygen uptake is apparently independent of the medium oxygen tension, but could be induced when roots are flooded. Auxin has been shown to accumulate and act as a master regulator of adventitious roots in flooded tomato hypocotyls (Vidoz et al., 2010). In addition, auxin has been recently shown to be involved as a modulator of mitochondrial functions and respiration (Batista-Silva et al., 2019; Berkowitz et al., 2016). However, reduced auxin transport with an auxin transport inhibitor (2,3,5-triiodobenzoic acid, TIBA) did not decrease oxygen consumption in submerged hypocotyls (Fig. S4). This lead us to believe that auxin is apparently not involved as an elicitor of respiration in stems of flooded plants. It is tempting to speculate that, once flooding stress is sensed at the root level, a systemic signal might be conveyed via xylem to shoots triggering this response (Else et al., 2006).

We proved that elevated oxygen consumption in flooded stems is indeed associated with a higher respiratory metabolism. Hexose kinases are considered the main gateway to glycolysis (Claeyssen and Rivoal, 2007) and their activity represent a major limiting

425 factor for carbon metabolism in submerged tomato roots (Gharbi et al. 2007; 2009).

426 Indeed, the glycolytic flux seems enhanced, for the activity of GK is high (Fig. 2A).

427 Generally, hypoxia sensing entails the shutdown of mitochondrial activity in plants

428 (Shingaki-Wells, 2014). However, the mitochondrial respiratory chain is highly active

429 in submerged hypocotyls as demonstrated by the reduction of TTC and the higher COX

430 and AOX capacity (Fig. 2B, C, D). Although it can be argued that the measurement of

431 COX and AOX capacity does not prove the actual engagement of each oxidase in

432 mitochondria (Day at al., 1996), it may be useful to reveal an intensified respiratory

433 metabolism in tissues (Zheng et al., 2021). ATP is usually considered limiting in organs

434 exposed to low oxygen (Cho et al. 2021) but, in submerged hypocotyls, its production is

435 boosted further confirming that respiration occurs at high rates in stems of flooded

436 tomato plants (Fig. 2E).

437 Subsequently, we asked whether submerged hypocotyls perceive the external hypoxic

438 condition. The expression of the alcohol dehydrogenase gene responds to low oxygen in

439 plants and could be considered a marker of hypoxia sensing (Klok et al., 2002).

440 *LeADH2* gene expression and ADH enzymatic activity in submerged hypocotyls (Fig.

441 S5A, B) indicate that the hypocotyl is able to sense hypoxia. It is therefore likely that,

442 although oxygen tension in flooding water does not restrain respiration possibly due to

443 the very high affinity of COX for oxygen (Geigenberger et al., 2000), it would be low

444 enough to elicit *LeADH2* gene expression. It could be objected that this apparently

445 wasteful use of oxygen in submerged tomato stems would inevitably lead to internal

446 anoxia with serious consequences for cell viability (Geigenberger, 2003). However, the

447 formation of aerenchyma facilitates the aeration of submerged hypocotyls and enables

448 this high rate of respiration (Mignolli et al., 2020).

449   Some carbon inputs are expected to sustain respiration in flooded hypocotyls. Starchy

450   reserves have been proved to be hydrolysed under hypoxia (Loreti et al., 2018).

451   Nevertheless, in our case, starch might not be used as carbon source since no

452   consumption was observed (Fig. 5C). Blocking phloem transport has been shown to

453   reduce carbohydrate allocation to roots and therefore their respiration (Högberg et al.,

454   2001; Walsh et al., 1987). Similarly, when the phloem sap supply to the submerged

455   hypocotyl is interrupted by girdling, the uptake of oxygen and the reduction of TTC

456   significantly declined (Fig. 3B) suggesting that leaf-derived sugars are involved. In

457   most plants, sucrose is the dominant carbohydrate that is transported via phloem (Liu et

458   al., 2012). In a recent paper, Takahashi et al. (2018) raised the question whether sucrose

459   has a metabolic or signalling role in triggering adaptive responses in flooded stems of

460   soybean. Our data pointed to sucrose as the main source of energy that is utilized by the

461   flooded hypocotyl since: i) sucrose promoted oxygen uptake more than other sugars

462   (Fig. 4A); ii) sucrose cannot be replaced by its metabolically inert analogue turanose,

463   which activates the sucrose signalling without triggering respiration (Fig. 4A); and iii) a

464   linear response between sucrose availability and respiration rate was observed (Fig. 4B).

465   If sucrose is required as a substrate for respiration, active cleavage into hexoses should

466   be therefore expected. Indeed, several clues indicate that sucrose cleavage takes place in

467   flooded tomato hypocotyls, for glucose and fructose are detected in relatively large

468   amounts (Fig. 5B) and the higher ratio hexose-to-sucrose suggests that sucrose is being

469   hydrolysed (Zrenner et al., 1996; Fig. S6). Consistently, *LIN6* and *SUS1* genes,

470   encoding an apoplastic invertase and a sucrose synthase respectively, were highly

471   upregulated in submerged hypocotyls (Fig. 4C, D). With respect to this observation,

472   both enzymes could be equally important for sucrose utilization under hypoxia,

473   especially when there is a high demand of carbohydrates (D'Aoust et al., 1999; Godt

474   and Roitsch, 1997; Santaniello et al., 2014).

475   Besides being consumed, phloem-derived sugars also accumulated in hypocotyls of

476   flooded tomato plants (5A, B). If sugars build-up in hypocotyls was merely the

477   consequence of a decreased sugar translocation to roots due to flooding (Saglio, 1985),

478   we would expect a similar effect in non-flooded plants when the sugar supply to roots is

479   cut off by girdling. However, neither respiration nor soluble sugars content increased in

480   low-girdled non-flooded plants while, they did so when roots were waterlogged (Fig.

481   5E). In fact, sugar accumulation in above-ground organs in response to flooding is

482   probably a well-coordinated mechanism encompassing reduced sugar export, sluggish

483   phloem flow and reduced sugars consumption at root level (Araki et al., 2012; Peuke et

484   al., 2015; Lothier et al., 2020).

485   We propose the existence of a connection between respiration and sugar storage.

486   Indeed, when hypocotyl respiration is inhibited with cyanide, sugar accumulation also

487   diminishes (Fig. 5F) suggesting that increased respiration is required to sustain sucrose

488   unload from phloem and its storage in hypocotyls (Milne et al., 2018).

489   Overall, our data indicate that, due to oxygen shortage in soil, root respiration and

490   growth are strongly hindered. Concomitantly, the energy demand for sugar unloading

491   and transport to hypocotyls would prompt respiration. As the availability of substrate

492   increases in flooded hypocotyls, respiration would be further fomented with the result of

493   'attracting' more sugars (Fig. 6).

494   The answer to the question whether or not the phenomenon has an adaptive significance

495   is not straightforward. Sugar storage in flooded plants has been suggested to be invoked

496   for its subsequent utilization when stress subsides (Albrecht et al., 2004). However, we

497   can also suppose that stored sugars act as a reserve to fulfil the energy requirements for

498   adventitious roots and aerenchyma formation (Fig. S3; Qi et al., 2020; Takahashi et al.,

499   2018), or cell expansion upon sucrose cleavage into hexoses (Mignolli et al., 2020;

500   González et al., 2005). The baffling question is why the same increase in respiration and

501   sugar accumulation occurs in waterlogged plants, where neither aerenchyma nor ARs

502   nor stem hypertrophy take place. A possible explanation could be that the formation of a

503   new sugar sink in the hypocotyl alleviates the feedback inhibition of photosynthesis

504   caused by root sink loss under flooding stress (Paul and Foyer, 2001). Plants ability to

505   withstand a changing environment has long fascinated scientists and we believe that our

506   observations further fuel our interest in understanding some of their most unexpected

507   behaviours.

508

509   **Author contributions**

510   F.M. and M.L.V. conceived the project. F.M. carried out oxygen measurements. F.M.

511   and J.O.B. conducted metabolites analysis. J.O.B. analysed the data. F.M. and M.L.V.

512   wrote the manuscript.

513

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519

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734

735

736 **Figure captions**

737

738 Figure 1

739 Root oxygen consumption rate and root biomass (A) in control and partially submerged

740 plants. Hypocotyl oxygen consumption rate in partially submerged and in waterlogged

59
60

741 plants (B). For oxygen consumption measurements in both roots and stems, data are the

742 mean of 4 replicates ± SD whereas for root biomass each point is the mean ± SD of 6

743 replicates from independent experiments. Statistical differences between control and

744 submerged/waterlogged plants were analysed with Unpaired t test (* $P < 0.05$; ** $P <$

745 0.01; *** $P < 0.001$; **** $P < 0.0001$).

746

747 Figure 2

748 Glucose kinase, GK, and fructose kinase, FK, enzymatic activity in control and

749 submerged hypocotyls (A). Data are the mean ± SD of 4 replicates. 2,3,5-

750 triphenyltetrazolium chloride (TTC) staining of hypocotyl sections from of control and

751 partially submerged plants (B). Vertical bars indicate 1 cm. Quantitative estimation of

752 formazan content in stained hypocotyl sections (C). Data are the means of 5 replicates

753 ±SD. Cytochrome and alternative oxidase capacity in control and submerged hypocotyls

754 (D). Each bar is the mean of 5 replicates ± SD. ATP content in control and submerged

755 hypocotyls (E). Each bar is the mean of 4 replicates ± SD. Statistical differences

756 between control and submerged hypocotyls in (A) and (D) were analysed with Unpaired

757 t test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). In (C), different letters

758 indicate statistical differences according to the non-parametric Kruskal-Wallis test ($P <$

759 0.05). For (E), different letters indicate statistical differences according to one-way

760 ANOVA with Tukey's HSD multiple comparison test ($P < 0.05$).

761

762 Figure 3

763 Effect of girdling on hypocotyls of control and partially submerged plants (A). Girdling

764 was carried out 1 cm above the cotyledonary node in 4-week-old plants (high girdling,

765 HG). Oxygen uptake rate (B, left histogram) and formazan content (B, right histogram)

766   in control, C, and partially submerged, S, plants. Plants were left intact "-" or high-

767   girdled "+". Each bar is the mean of 4 to 5 replicates ± SD. Different letters indicate

768   statistical differences according to one-way ANOVA with Tukey's HSD multiple

769   comparison test (P < 0.05). In all experiments, data were taken after 3 days of partial

770   submersion.

771

772   Figure 4

773   Oxygen consumption rate in sugar-fed hypocotyls (A). Hypocotyls of control plants

774   were incubated in presence of no sugars, C, and equimolar (20 mM) concentrations of

775   glucose, Glc; fructose, Fru; sucrose, Suc; maltose, Mal and turanose, Tur. Each bar is

776   the mean of 6 replicates ± SD. Sucrose concentration-dependent oxygen consumption

777   assay (B). For each sucrose concentration, the oxygen uptake of 5 hypocotyls was

778   measured and a linear regression was calculated. Relative transcription level of

779   apoplastic invertase *Lin6* (C) and sucrose synthase *Sus1* gene (D) in control and

780   submerged hypocotyls. Each bar represents the mean of 3 replicates ± SD. For each

781   gene, the expression of control hypocotyl at the beginning of the experiment was set

782   arbitrarily to 1. For graphs (A), (C) and (D) different letters indicate statistical

783   differences according to one-way ANOVA with Tukey's HSD multiple comparison test

784   (P < 0.05).

785

786   Figure 5

787   Content of total soluble sugars in hypocotyls of control, partial submerged and

788   waterlogged plants (A). Each bar is the mean ± SD of 4 replicates. Sucrose, fructose,

789   glucose (B) and starch content (C) in hypocotyls of control and flooded plants. Each

790   point is the mean ± SD of 4 replicates. Total soluble sugars in hypocotyl of girdled

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flooded plants (D). Partial submerged plants were left intact or girdled above cotyledons

the day before partial submergence (high girdling, HG). Flooding experiments lasted 3

days. Each bar is the mean ± SD of 4 replicates. Oxygen consumption rate and total

soluble sugars (E) in hypocotyls of intact control, C, intact waterlogged, W, and low-

girdled non-flooded plants, LG. Each bar is the mean ± SD of 5 replicates. Effect of

KCN on oxygen uptake and sugar content (F) in hypocotyls of partially submerged

plants. Four-week-old plants were submerged up to cotyledonary node in 0.5 mM KCN

solution and in pure water as control for 3 days. KCN solution was renewed daily. Each

bar is the mean ± SD of 5 replicates. Data in graphs A and C were analysed with non-

parametric Kruskal-Wallis test ($P < 0.05$). For graphs B and E, different letters indicate

significant differences according to one-way ANOVA with Tukey's HSD multiple

comparison test ($P < 0.05$). Statistical analysis in figures D and F was carried out using

the Unpaired t test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

Figure 6

Schematic diagram proposing the possible mechanism that leads to increased respiration

and sugar accumulation in flooded tomato stems. Flooding causes soil hypoxia, which

dampens root respiration and ultimately causes growth arrest. Possibly, a yet

unidentified signal from hypoxic roots induces phloem-derived sucrose unloading in the

submerged hypocotyl. Increased availability of substrate (sucrose) fuels further

respiration which in turn sustains additional sugar unloading in the hypocotyl, leading to

the establishment of a storing tissue.