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1 Ethylene seems required for the berry development and ripening in grape, a non-
2 climacteric fruit.

3

4 Christian Chervin*, Ashraf El-Kereamy¹, Jean-Paul Roustan, Alain Latché, Julien Lamon and
5 Mondher Bouzayen

6

7 UMR990 INRA/INP-ENSAT, Avenue de l'Agrobiopole, BP 107, 31326 Castanet-Tolosan,
8 France

9

10 *Corresponding author: Christian Chervin, ENSAT BP 107, 31326 Castanet, France

11 Ph/Fax: +33 5 62 19 35 73

12 email: chervin@ensat.fr

13

14 ¹Present address: Department of Horticulture, Faculty of Agriculture, Ain Shams University,

15 P.O. Box: 68, Hadayek Shoubra, 11241 Cairo, Egypt

1 **Abstract:**

2

3 While the grape has been classified as a non-climacteric fruit whose ripening is
4 thought to be ethylene independent, we show here that a transient increase of endogenous
5 ethylene production occurs just before veraison (i.e. inception of ripening). We observed that
6 ethylene perception, at this time, is required for at least the increase of berry diameter, the
7 decrease of berry acidity and anthocyanin accumulation in the ripening berries; these latter
8 experiments were performed with 1-methylcyclopropene, a specific inhibitor of ethylene
9 receptors. The potential roles of ethylene in berry development and ripening are discussed.

10

11 **Keywords:** grapes, *Vitis vinifera*, ethylene, ripening, non-climacteric

12

13 **Abbreviations:** 1-MCP, 1-methylcyclopropene; ACC, 1-aminocyclopropane-1-carboxylic
14 acid; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase.

1 **1. Introduction**

2

3 Three facts led us to check the influence of endogenous ethylene and active receptors
4 in development and ripening phases of grape berries: (i) earlier observations showing that
5 grape ripening can be either inhibited or promoted by exogenous ethylene, depending on the
6 application time over the berry development period (Hale et al. 1970); (ii) the observation of a
7 peak of ethylene production around veraison (Alleweldt and Koch, 1977), and (iii) the
8 availability of 1-methylcyclopropene (1-MCP), a specific inhibitor of ethylene receptors
9 (Blankenship and Dole, 2003).

10 Although in the 1970's ethylene was thought to have a very limited role, if any, in the
11 ripening process of non-climacteric fruit (Coombe and Hale, 1973; Abeles et al., 1992), more
12 recent works have revealed that some aspects of non-climacteric ripening may be associated
13 with ethylene responses (Giovannoni, 2001). The classification of grapes as non-climacteric
14 fruit was mainly due to a set of data showing only weak changes in endogenous ethylene
15 levels around veraison (Coombe and Hale, 1973), a development stage at which grape berries
16 start to loose their acidity and to redden, in the case of red cultivars, among other biochemical
17 changes. Indeed, Coombe and Hale (1973) and Alleweldt and Koch (1977) found that the
18 amounts of endogenous ethylene produced by grapes were quite small when expressed as a
19 concentration per volume of internal gas (less than $0.5 \mu\text{l.l}^{-1}$), but when expressed as a
20 concentration per weight of tissue, then an ethylene burst was clearly observable around
21 veraison (Alleweldt and Koch, 1977). However in this latter study, the peak was made of one
22 point only (one date at which the ethylene production rose), and the fruit was incubated for
23 one hour under partial vacuum, an excessive period of time over which some of the ethylene
24 collected could be a part of plant responses to vacuum.

25

1 **2. Materials and methods**

2

3 *2.1. Plant material and 1-MCP treatments*

4 Cabernet sauvignon grapevines are grafted on 110 Richter rootstocks and grown in
5 Toulouse, South-West of France, in a non-irrigated vineyard. The observations were
6 performed over two consecutive years; the full bloom occurred around mid-June. The 1-MCP
7 was applied at various times following full bloom, for a 24 hour period, in a polyethylene bag
8 wrapped around the cluster, at an initial concentration of 4 $\mu\text{l.l}^{-1}$. Control clusters were
9 wrapped into plastic bags for 24 h. For these experiments, clusters growing in a shaded area
10 of the vines were chosen to avoid direct exposure to sunlight and overheating associated with
11 such a treatment. After the 24 hour periods of treatment, the clusters were sampled and
12 assayed immediately for ACO activity and juice acidity or stored at -80°C .

13

14 *2.2. Measurement of internal ethylene*

15 The internal ethylene was assessed according to Coombe and Hale (1973). Briefly,
16 control whole clusters that had not been incubated in plastic bags, weighing a total of 150 g
17 approximately, were placed in a bowl filled with a NaCl solution at saturation, under an
18 inverted funnel with an exhaust blocked by a rubber septum. The air remaining in the funnel
19 exhaust was taken out with a syringe. Then the bowl was incubated under a partial vacuum of
20 -700 mm Hg for 5 min, in a freeze-dryer chamber. After returning to atmospheric pressure
21 one ml of the internal atmosphere caught in the funnel under the septum was sampled with a
22 syringe and injected in a gas chromatograph.

23

24

25

1 2.3. *Assay of ACO activity and ACC content*

2 The *in vivo* ACO activity was assayed using one gram _{FW} of berry halves for 1.2 ml
3 of *in vivo* buffer described by Pretel et al. (1995), with the following modifications: Tris-HCl
4 0.5M, pH 7 and mannitol 0.35 M. The berry content of 1-aminocyclopropane-1-carboxylic
5 acid (ACC) was assayed according to Mansour et al. (1986).

6

7 2.4. *Northern blot analysis*

8 Northern blots were performed according Boss et al. (1996). The corresponding
9 cDNA probe was obtained from genomic grape DNA using sequences with GenBank
10 accession number AY211549. The probe matched a 255 bp sequence of the coding region at
11 the 3' end.

12

13 2.5. *Assessment of berry growth, acidity of the juice and anthocyanin content of the skin*

14 The diameter was assessed using callipers as described by Coombe (1992). The
15 titratable acidity of the juice was measured with 0.1 N NaOH up to pH 7. The total
16 anthocyanin content was assayed according to Boss et al. (1996), and converted to malvidin-
17 3-glucoside equivalents using a ϵ of 28,000 $\text{Mol}^{-1}.\text{cm}^{-1}$ at 520 nm (Souquet J.M., pers.
18 comm.).

19

20 2.6. *Statistical analysis*

21 In order to determine the LSDs at the 0.05 level, analyses of variance were performed
22 with SigmaStat (SPSS Inc., Chicago, IL).

23

24

25

1 3. Results and Discussion

2

3 3.1 Ethylene production in developing berries

4 In our observations (Figure 1a), we confirmed the occurrence of this ethylene peak
5 in Cabernet Sauvignon grape clusters (*Vitis vinifera*, L.) and observed the rise in ethylene
6 production over more than one date (weeks 6, 7 and 8), using only five minutes of gas
7 collection under vacuum. This peak represents a concentration around $0.2 \mu\text{l.l}^{-1}$, which is
8 above the physiological threshold in most plant tissues (Abeles et al., 1992). In the same
9 grapes, we monitored *in vivo* activity (Figure 1a) and transcript accumulation (Figure 1b) of
10 an 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), the last enzyme in the ethylene
11 production pathway, and both matched the occurrence of the ethylene peak. Additionally, the
12 pre-veraison ethylene peak was observed over two consecutive years, in irrigated and non-
13 irrigated Cabernet Sauvignon vineyards, one with 110 Richter and the second with 3309
14 Couderc rootstocks. However, the peak was more or less advanced depending on the climatic
15 conditions of the preceding month in each year (data not shown), and we reproduce here the
16 data of one year only. Additionally, the content of total 1-aminocyclopropane-1-carboxylic
17 acid (ACC), the immediate precursor of ethylene, including conjugated and free forms,
18 reached levels that were 20 times higher than those of the free form alone (Figure 1c). This
19 means that most of the ACC was malonylated, and suggests that in grapes the competition for
20 ACC between ACO and ACC malonyl transferase described previously (Mansour et al.,
21 1986), is in favour of the latter. The levels of total ACC reached approximately 5 nmoles of
22 per gram of fresh weight at veraison, 1000 times greater than the levels of ethylene
23 production, suggesting that the ACC production was not limiting. This high ACC content in
24 grapes had already been noticed in a previous work (Mizutani et al., 1988). The slight delay
25 between the ethylene peak (week 7) and the ACC peak (week 8) can be explained by the time

1 necessary to the berry tissues to accumulate high levels of ACC. The decrease in ACC levels
2 per gram of fresh weight at weeks 9 and 10 can be explained by the restart of berry growth
3 after veraison (Coombe and McCarthy, 2000).

4

5 *3.2. Importance of the ethylene perception on the berry physiology*

6 In order to check whether this temporary rise in ethylene production has some
7 physiological importance on grape ripening, we blocked ethylene receptors with 1-MCP at
8 different times around the expected ethylene peak (i.e. 5 to 9 weeks after full bloom). 1-MCP
9 is a gas at ambient temperature and atmospheric pressure; it has been described as an
10 irreversible inhibitor of ethylene receptors, with an affinity for the receptors 10 times greater
11 than that of ethylene (Blankenship and Dole, 2003).

12 As shown in Figure 2a, we observed that application of 1-MCP delayed the increase of
13 berry diameter. This delay was correlated to the application of 1-MCP at the time of the
14 ethylene peak (Figure 1a). According to Coombe and McCarthy (2000), at the beginning of
15 the second growth phase, berry growth is mainly linked to phloem fluxes, but it is not
16 excluded that some sap comes from xylem tissues. The roles of ethylene on these fluxes are
17 not well described in the literature. However the ethylene seems to have a role in cell
18 enlargement (Sanchez-Calle et al., 1989; Camp et al., 1981). This role could explain the
19 limitation of diameter increase due to the blockage of ethylene receptors by 1-MCP.

20 Additionally, the results of Figure 2b suggest that ethylene may affect the acidity
21 decrease that is a feature of the post-veraison period of grape ripening. Grapes treated with 1-
22 MCP at 6, 7 and 8 weeks after full bloom had higher acidity levels than untreated controls
23 when harvested at 13 weeks post bloom. The strongest MCP effects were seen for treatments
24 that corresponded with the timing of the endogenous ethylene peak. At this time of berry
25 development, the decrease in juice acidity is explained mainly by the decrease of the malic

1 acid concentration (Ollat et al., 2002). This decrease can be itself induced by ethylene as part
2 of the increased respiration known to be triggered by this phytohormone even in non-
3 climacteric tissues (Abeles et al., 1992). Indeed, Saulnier-Blache and Bruzeau (1967) showed
4 that several grape cultivars underwent an increase in CO₂ evolution at veraison that could be
5 part of a respiratory burst. It was associated to a lesser extent with a rise in O₂ uptake. This
6 respiratory rise lasted for at least a fortnight following veraison (after which the
7 measurements were stopped), and it seems to match the period of acidity drop of the berry
8 juice. Other authors have suggested that malic enzyme could also be activated at veraison and
9 be part of malate catabolism (Ollat et al., 2002), and this enzyme has also been shown to be
10 inducible by ethylene in ripening fruit (Mamedov et al., 1997). Moreover, the transport of
11 organic acids within cell compartments is obviously involved in acid metabolism (Terrier and
12 Romieu, 2001) and this transport may be modulated by ethylene signals (Schmidt et al.,
13 2003). However, it cannot be ruled out that the sustained acidity (Fig. 2b) could simply
14 result from the inhibited fruit expansion (Fig. 2a).

15 Finally, 1-MCP was also shown to transiently inhibit anthocyanin accumulation in
16 berry skins (Figure 2c). Again this inhibition was stronger when the 1-MCP was applied at the
17 time of the ethylene peak. This is less surprising, as the expression of several enzymes of the
18 anthocyanin pathway (Robinson and Davies, 2000) can be induced by ethylene signals (El-
19 Kereamy et al., 2003). It is also possible that impaired fruit expansion might have an effect on
20 other signals leading to anthocyanin synthesis and accumulation, i.e. sugar levels (Vitrac et
21 al., 2000). Indeed, it is known that sugar accumulation in berries starts around veraison and is
22 linked to phloem unloading (Coombe and McCarthy, 2000).

23 Such 1-MCP experiments have been conducted over two consecutive years and similar
24 results have been observed. The results presented here are the data set of a single year,
25 because the time at which the sensitivity to 1-MCP is maximal depends on the climate in the

1 month following bloom, that also impacts on the ethylene peak. In these experiments (Figure
2), the berries were picked a few weeks before harvest as we noticed in preliminary trials that
3 treated grapes can overcome the 1-MCP inhibition of ripening as time goes by, may be
4 through *de novo* synthesis of ethylene receptors.

5
6 Our observations regarding the role of internal ethylene in modulating some
7 metabolisms associated with berry development and ripening in grapes, confirm what other
8 researchers observed with applications of exogenous ethylene. Indeed, Hale et al. (1970) and
9 others (Weaver and Montgomery, 1974; Shulman et al., 1985) observed that these
10 applications enhanced acidity drop and the accumulation of red pigments. This suggested that
11 the berry tissues were able to sense ethylene, but in the 1970's nothing was known about
12 ethylene signal transduction. Since then, commercial treatments with ethylene precursors have
13 been developed, but these precursors are applied at rate that should give rise to more than 500
14 $\mu\text{l.l}^{-1}$ of ethylene internal concentration if every mole of the precursor penetrates the plant
15 tissues and is transformed to ethylene. So several researchers suggested that such treatments
16 are performed at too high concentrations to give a physiological meaning to the plant response
17 to this ethylene treatment, however such treatments give rise to concentrations of internal
18 ethylene that are 100 times smaller than expected (El-Kereamy et al., 2003).

19 One could argue that the ripening delay induced by 1-MCP was only due to a toxic
20 effect of this molecule. However two facts can be raised against this argument: (i) the changes
21 induced by 1-MCP are contrary to those induced by exogenous ethylene (Weaver and
22 Montgomery, 1974; Shulman et al., 1985); (ii) the same 1-MCP dose had no effect on the
23 berry physiology (i.e. no toxic effect) if applied before or after the ethylene peak, when it
24 delayed the berry ripening if applied at the time of the ethylene peak (Figure 2).

1 We have not yet characterised the responses to 1-MCP in other cultivars than Cabernet
2 Sauvignon, but similar responses are expected knowing that many cultivars respond similarly
3 to exogenous ethylene (Weaver and Montgomery, 1974; Shulman et al., 1985).

4 5 **3. Conclusion**

6 Obviously, the grapes contain a functional network of ethylene signalling at the onset
7 of ripening, and part of this complex is necessary to the ripening process. Our data do not
8 imply that grape should be considered as a climacteric fruit, but that new techniques and new
9 tools may change the way of categorising fruit ripening. Further interesting studies are
10 granted, particularly with the development of grape micro-arrays. These studies will bring
11 new insights into the triggering events of ripening metabolism of non-climacteric fruit.

12 13 14 **Acknowledgements:**

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1 **Figure captions**

2

3 **Figure 1:** **a)** Changes in internal ethylene of Cabernet Sauvignon clusters and changes in the
4 *in vivo* ACO activity of the berry tissues as a function of the time after full bloom; n = 3,
5 error bars show SE. **b)** Changes in ACO transcript accumulation in berries as a function of
6 the time after full bloom. **c)** Changes in 1-aminocyclopropane-1-carboxylic acid (ACC)
7 levels in berries as a function of the time after full bloom; n = 3, error bars show SE.

8

9 **Figure 2:** Influence of gassing Cabernet Sauvignon clusters at various times after full bloom
10 with 1-methylcyclopropene (1-MCP), ethylene competitive inhibitor, on three maturity
11 parameters of berries harvested 13 weeks after full bloom; **a)** diameter, **b)** titratable acidity of
12 the juice and **c)** anthocyanin content of the skins. The data are means of 3 replicates \pm
13 standard errors and LSDs were determined at the 0.05 level.

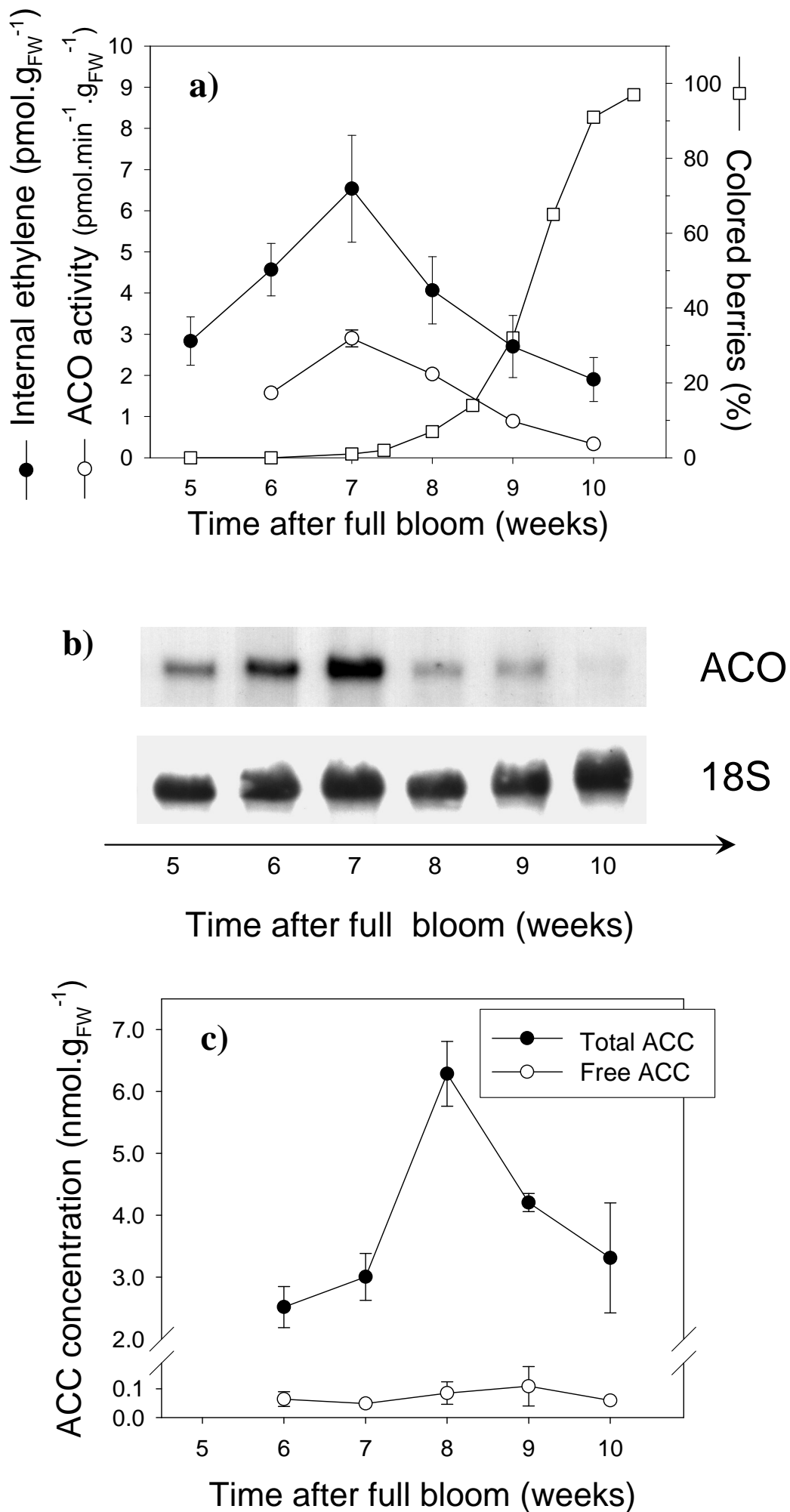


Figure 1

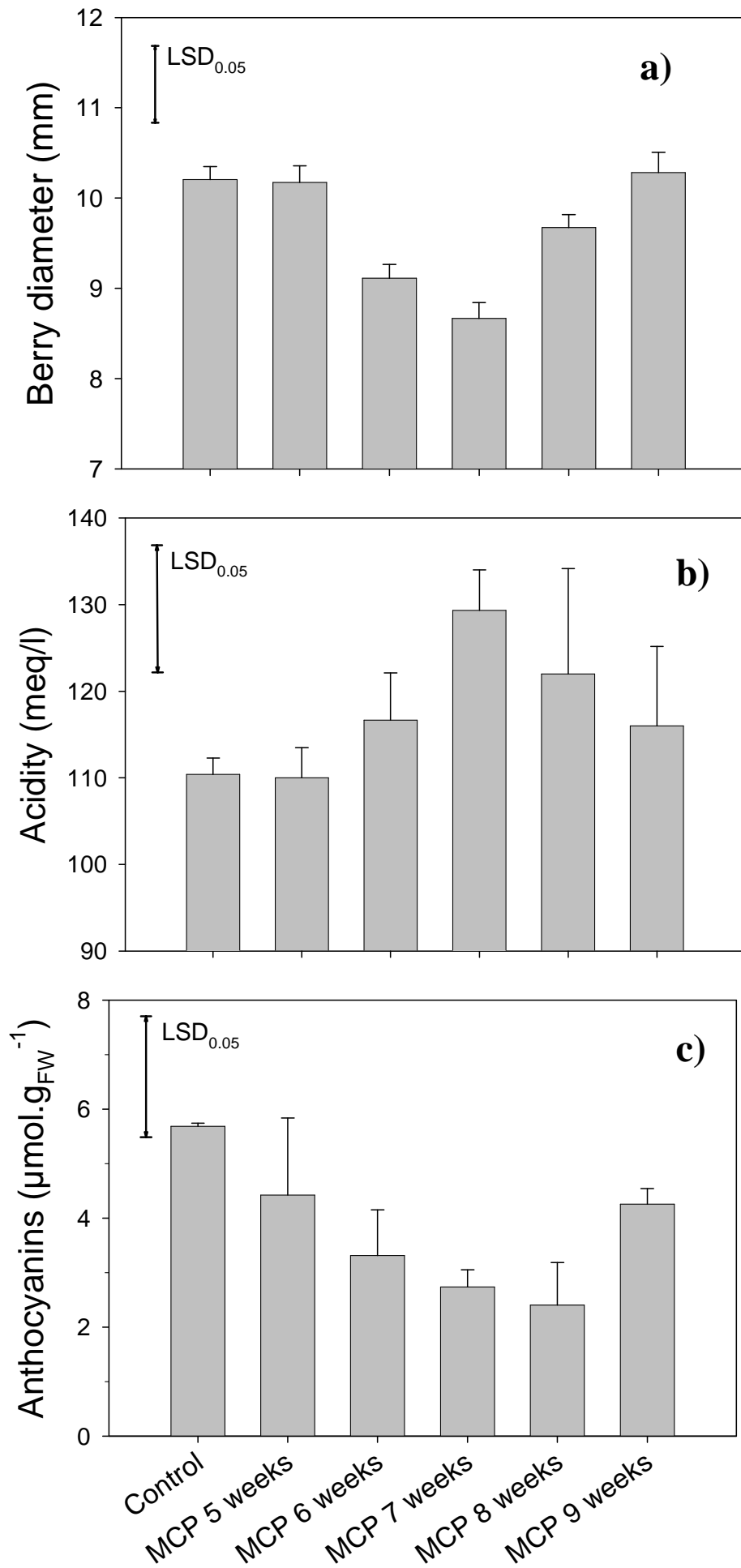


Figure 2