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

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Abstract:

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

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

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

1. Introduction

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

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

2. Materials and methods

2.1. Plant material and 1-MCP treatments

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

2.2. Measurement of internal ethylene

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

2.3. Assay of ACO activity and ACC content

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

2.4. Northern blot analysis

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

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

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

2.6. Statistical analysis

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

3. Results and Discussion

3.1 Ethylene production in developing berries

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

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

3.2. Importance of the ethylene perception on the berry physiology

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

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

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

acid concentration (Ollat et al., 2002). This decrease can be itself induced by ethylene as part of the increased respiration known to be triggered by this phytohormone even in non-climacteric tissues (Abeles et al., 1992). Indeed, Saulnier-Blache and Bruzeau (1967) showed that several grape cultivars underwent an increase in CO_2 evolution at veraison that could be part of a respiratory burst. It was associated to a lesser extent with a rise in O_2 uptake. This respiratory rise lasted for at least a fortnight following veraison (after which the measurements were stopped), and it seems to match the period of acidity drop of the berry juice. Other authors have suggested that malic enzyme could also be activated at veraison and be part of malate catabolism (Ollat et al., 2002), and this enzyme has also been shown to be inducible by ethylene in ripening fruit (Mamedov et al., 1997). Moreover, the transport of organic acids within cell compartments is obviously involved in acid metabolism (Terrier and Romieu, 2001) and this transport may be modulated by ethylene signals (Schmidt et al., 2003). However, it cannot be ruled out that the sustained acidity (Fig. 2b) could simply result from the inhibited fruit expansion (Fig. 2a).

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

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

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 treated grapes can overcome the 1-MCP inhibition of ripening as time goes by, may be through *de novo* synthesis of ethylene receptors.

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

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

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

3. Conclusion

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

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Figure captions

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

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

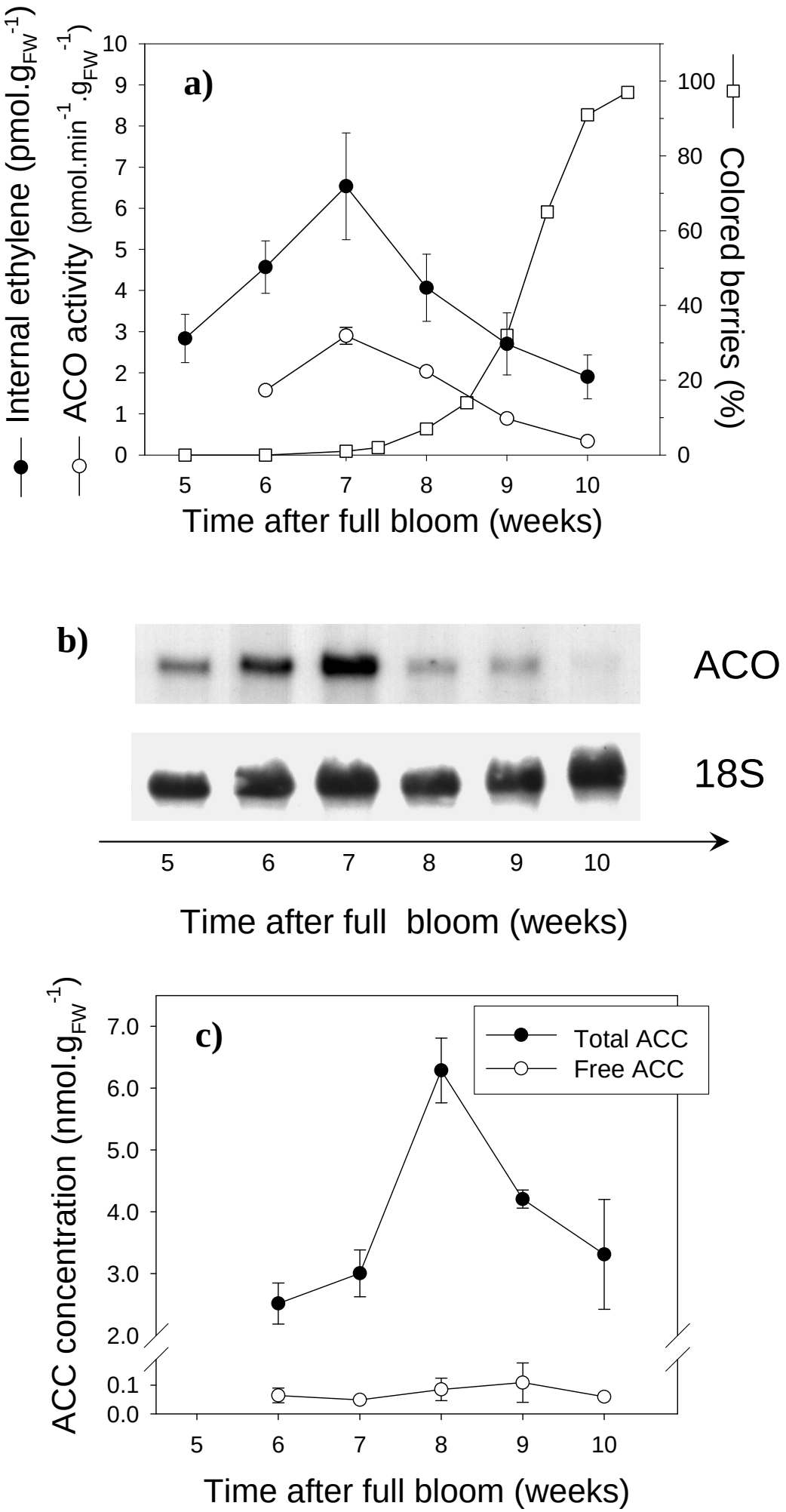
a)
Internal ethylene (pmol.g$_{FW}^{-1}$)
ACO activity (pmol.min^{-1}.g$_{FW}^{-1}$)
Colored berries (%)
Time after full bloom (weeks)
b)
ACO
18S
Time after full bloom (weeks)
c)
Total ACC
Free ACC
ACC concentration (nmol.g$_{FW}^{-1}$)
Time after full bloom (weeks)

Figure 1

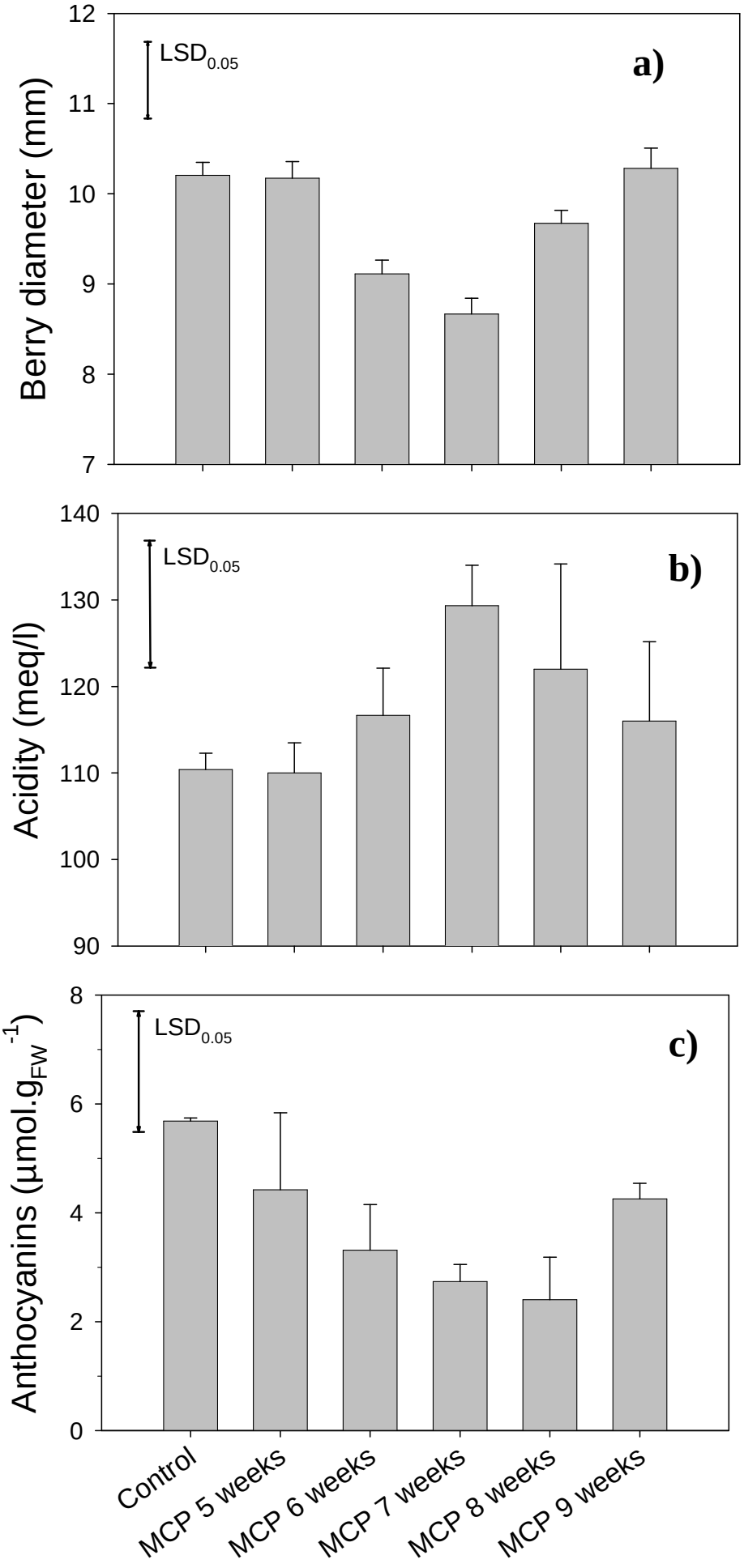
a)
b)
c)
LSD0.05
Berry diameter (mm)
Acidity (meq/l)
Anthocyanins (µmol.gFW-1)
Control
MCP 5 weeks
MCP 6 weeks
MCP 7 weeks
MCP 8 weeks
MCP 9 weeks

Figure 2