Involvement of ethylene signalling in a non-climacteric fruit: new elements regarding the regulation of *ADH* expression in grapevine

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Abbreviations: ARE: anaerobic responsive element; CEPA: chloroethylphosphonic acid; ERE: ethylene responsive element; MCP: methylcyclopropene, UTR: untranslated

region

Abstract

Although grape berries have been classified as non-climacteric fruits, ongoing studies on grape ethylene signalling lead to challenge the role of ethylene in their ripening. One of the significant molecular changes in berries is the up- regulation of ADH (alcohol dehydrogenase, EC. 1.1.1.1) enzyme activity at the inception of fruit ripening and of *VvADH2* transcript levels. This paper shows that the ethylene signal transduction pathway could be involved in the control of *VvADH2* expression in grapevine berries and in cell suspensions. The induction of *VvADH2* transcription, either in berries at the inception of ripening or in cell suspensions, was found to be partly inhibited by 1-methylcyclopropene (1-MCP), an inhibitor of ethylene receptors. Treatment of cell suspensions with 2-chloroethylphosphonic acid (2-CEPA), an ethylene releasing compound, also resulted in a significant increase of ADH activity and *VvADH2* transcription under anaerobiosis, showing that concomitant ethylene and anaerobic treatments in cell suspensions could result in changes of *VvADH2* expression. All these results, associated with the presence in the *VvADH2* promoter of regulatory elements for ethylene and anaerobic response, suggest that ethylene transduction pathway and anaerobic stress could be in part involved in the regulation of *VvADH2* expression in ripening berries and cell suspensions. These data open new aspects of the expression control of a ripening-related gene in a non-climacteric fruit.

Key words: Alcohol dehydrogenase, anaerobiosis, anoxia, fruit development, ethylene, 1-methylcyclopropene (1-MCP), suspension cells, *Vitis vinifera*.

Introduction

Much progress has been made in recent years in the identification of changes at the molecular level occurring during grape berry ripening. In *Vitis vinifera L.*, both the transcription of the *VvADH2* gene and ADH enzyme activity are up-regulated during berry ripening (Tesniere and Verries, 2000; 2001). Several other events have been shown to occur during berry development (Boss and Davies, 2000; van Heeswijck et al., 2000). Despite this progress, it remains largely unknown how the signal that triggers fruit ripening is transduced in grape berries. Fruits in which the production of ethylene increases strongly during the ripening phase with a peak in respiration are classified as climacteric fruit. Grape berries that do not accumulate ethylene to the same extent and lack the peak in respiration are thus classified as non-climacteric fruits (Coombe and Hale, 1973). However, fruit ripening is considered to involve both ethylene-dependent and ethylene-independent processes (Lelievre et al., 1997). In fact, a limited production of ethylene has been described in grape berries (Alleweldt and Koch, 1977), as well as in other non-climacteric fruits (Hartmann and Boudot, 1988; Yeekwan et al., 1998). Recent work (Chervin et al., 2004) has shown that even though their ethylene production is low, grape berry ripening is impaired by 1-methylcyclopropene (1-MCP), a specific inhibitor of the ethylene receptor (Blankenship and Dole, 2003). In addition, the partial involvement of ethylene in anaerobic induction of *ADH1* in *Arabidopsis* seedlings has recently been reported (Peng et al., 2001).

It was therefore interesting to investigate whether the ethylene signal transduction pathway could be involved in the control of *ADH* expression in grapevine. The effect of 1-MCP on the ADH enzyme activity and *VvADH* transcript levels, both during berry development and in suspension cells of *V. vinifera* was thus compared. In addition, treatments were performed with 2-chloroethylphosphonic acid (CEPA), a chemical that releases ethylene when applied to plants, either alone or in combination with 1-MCP.

Material and methods

Plant material and treatments

Berries from grapevines (*V. vinifera* L., cv Cabernet Sauvignon) grown in a Toulouse vineyard (South-West of France) were used for all experiments. Clusters, wrapped in a

polyethylene bag, were exposed to 4 ppm gaseous 1-methylcyclopropene (1-MCP) for 24 h. This application was performed weekly, starting at week 6 post-flowering up to week 10. Single 24 h applications performed at week 6 to 10 prior to berry sampling (Single MCP) were compared to 5 applications (week 6 to 10) repeated on the same cluster (Repeated MCP). For example, the treatment called "Repeated" sampled at week 8 had been treated three times (once a week) for 24 h each. This latter treatment was performed to take into account any *de novo* synthesis of ethylene receptors. Three replicates were performed using three clusters (each one from a different vine) at a similar stage of development. After sampling, berries were frozen at –80 °C until further analysis.

Cell suspensions of *V. vinifera* cv Cabernet Sauvignon were grown as previously described (Torregrosa et al., 2002). Four-day-old subcultures were treated with 1-MCP and/or CEPA, 2-chloroethylphosphonic acid, a chemical that releases ethylene when applied to plants (Abeles et al., 1992). The 1-MCP treatment consisted in a single application of 1 ppm in the headspace of an erlenmeyer flask for 2 h. In some experiments, 50 µM CEPA was added to the cell suspension medium 2 h after the 1-MCP gassing. After treatment, 6 x 4 ml aliquots were incubated in 6-well microplates and were then either maintained in air or incubated under pure nitrogen for 24 h. Gas samples were withdrawn from the flask and analyzed by gas chromatography. Cell samples were collected on Whatman filters by vacuum filtration and immediately frozen in liquid nitrogen or placed in the appropriate extraction buffer. All experiments were repeated once. The results of the northern blots and activity assays performed on cell suspensions were expressed as values relative to the untreated control, to overcome variation between cultures repeats.

Refractive index and ethylene measurements

Sugar contents were evaluated using refractive index measurements, determined from supernatants of powdered frozen fruits. To insure that ethylene concentrations were approximately in the same range under similar treatment conditions, gas samples were taken from the headspace of the erlenmeyer flasks and ethylene was quantified. The jars were sealed for 2 h with a silicon cap placed over three layers of parafilm. The ethylene concentration in the jars was analyzed at the end of the treatment period by removing

a 20 ml gas sample through the seal. The samples were stored in a 25 ml penicillin tube at room temperature until assayed by gas chromatography using a FID (flame ionization detector) equipment and an alumina column (Mansour et al., 1986).

Protein and ADH enzyme activity measurements.

The frozen powder (0.1 g) of berry tissues was thawed in 0.3 ml of extraction buffer containing 0.2 M Bis-tris propane-MES (pH 8.0), 5 mM EDTA, 10% glycerol (p/v), 1% PVP (p/v), 1% Triton X-100 (v/v), 1 mM PMSF and 10 mM β-mercaptoethanol. After centrifugation (13,000 x *g*, for 5 min at 4 °C), supernatants were stored at -80°C until assayed. Cell suspension cultures were sampled by filtering 1 ml aliquots of the suspension culture on a 45 mm-diameter filter under vacuum. Collected cells were immediately transferred to 0.4 ml extraction buffer (0.1 M sodium phosphate, pH 7.8, 1 mM DTT, 0.1% Triton X-100 v/v). After sonication for 20 sec, lysates were centrifuged at 13,000 x *g* for 5 min at 4 °C and aliquots of the supernatants were stored at –80 °C until assayed. The Bradford method (Bradford, 1976) was used for all protein determinations, using BSA as standard. ADH activity was assayed by measuring the reduction rate of acetaldehyde at 340 nm as previously described (Molina et al., 1987; Tesniere and Verries, 2000).

Isolation of RNA and expression analysis

Total RNA was isolated from berries according to Boss et al. (1996), and from cell cultures using the SV total RNA isolation system from Promega (Madison, WI). Ten micrograms RNA per lane were fractionated on 1.2% formaldehyde agarose gels, blotted onto nylon membranes and hybridised to different ^{32}P-labelled 3' UTR *VvADHs* probes to specifically detect the different isogenes expressed in berries. Hybridisations were performed at 65 °C in 5X SSC, 5X Dendhardt's solution, 0.5% SDS, with 100 µg/ml denaturated salmon sperm DNA. Membranes were washed at high stringency. The hybridisation signals were quantified by direct scanning of the membranes and signal intensities were analyzed using a Storm imager (Molecular Dynamics, Sunnyvale, CA, USA). Normalization was achieved using the respective 18S ribosomal RNA values for each sample. The resulting data are relative ratios allowing the comparison between the intensities of different hybridisation signals on the same membrane.

Statistical analyses

Two-way ANOVAs were performed using SigmaStat v.2.0 (SPSS, Chicago). LSD values for each factor and the interaction were calculated at the 0.05 significance level.

Results

Developmental induction of ADH during berry ripening is affected by 1-MCP

The effect of 1-MCP on ADH activity was evaluated as shown in Fig. 1. A single treatment with 1-MCP had a significant effect on enzyme activity only in berries treated 10 weeks after flowering. Following repeated treatments, a reduction of enzyme activity was significant 9 weeks after flowering. In control samples, ADH activity increased during ripening as observed previously by Tesniere and Verries (2000). Measurement of the refractive index in control samples showed an accumulation of sugars from the onset of ripening at week 8 (data not shown), coinciding with the increase in ADH activity.

Northern-blot analyses were also conducted to check the expression pattern of *VvADH* isogenes. No significant levels of *VvADH1* and *VvADH3* transcripts were detected, in either treated or non-treated berries (data not shown). In contrast, the low expression of *VvADH2* observed in control berries up to the seventh week post-flowering, increased strongly and steadily thereafter (Fig. 2A). These data confirmed the pattern of expression of *VvADH2* during fruit development and that it is the predominant *ADH* isogene expressed in ripening berries (Tesniere and Verries, 2000). MCP applications significantly reduced the accumulation of *VvADH2* mRNAs (Fig. 2A), particularly 10 weeks after flowering. Figure 2B shows more clearly that the differences induced by the 1-MCP treatments were initiated after week 7 for repeated treatments or week 8 for single treatments. The weekly repeated application of 1-MCP resulted in a significant limitation of the up-regulation of *VvADH2* expression during ripening, varying from 30% to 50% of the control level. These results suggest that it is likely that the effect of 1-MCP varies with the physiological stage of the fruit. It may also vary with the treatment method. In any case, the results presented here suggest an inhibitory effect of 1-MCP on *VvADH* gene expression in ripening grape berries.

The reduction in ADH activity was generally smaller than the reduction observed at the transcript level, and observed with a one-week delay compared to the changes of *VvADH2* mRNAs. This difference could be due to a rapid *VvADH2* mRNAs turnover,

whereas ADH protein stability could account for a delay in the reduction observed at the mRNA level, as previously suggested (Tesniere and Verries, 2000). Finally, it is not excluded that part of the ADH activity is constitutive and not related to the ethylene signaling pathway.

Altogether these results suggest that an ethylene signal could modulate the induction of the *VvADH2* gene depending on the developmental stage of the fruit.

1-MCP and CEPA treatments alter ADH gene expression and enzyme activity in suspension cells

To investigate whether the previously observed effect of ethylene was fruit specific, the effects of 1-MCP and CEPA on suspension cells were also studied. The ethylene released in the head-space of CEPA treated cultures reached an average of 3 ± 1 ppm after a 24 h-incubation (Table 1). For all other cell cultures not treated with CEPA, the ethylene levels were under the physiological threshold of 0.1 ppm (Abeles et al., 1992). Compared to the control in air, ADH activity (Fig. 3A) reached levels that were 16% higher in CEPA samples, 48% higher in N_2 and 190% higher in CEPA + N_2. The same trend was observed for *VvADH2* mRNA levels (Fig. 3B). Compared to the control in air, the *VvADH2* transcripts reached levels that were 50% higher in CEPA samples, 50% higher in N_2 and 390% higher in CEPA + N_2. The 1-MCP effect observed in cell suspensions paralleled the results obtained with grape berries. The difference in amplitude of the response in ADH activity and *VvADH2* transcription may indicate that post-transcriptional regulation mechanisms are also involved.

Ethylene response elements are present in the VvADH2 promoter.

VvADH2 promoter sequence analysis revealed several core consensus sequences of putative ethylene (ERE, Montgomery et al., 1993; Itzhaki et al., 1994) and anaerobic responsive elements (ARE; GT-motif; Walker et al., 1987; Tesniere and Verries, 2001) as presented in figure 4. The sequence of the *VvADH3* promoter is unknown yet, but the 0.3 kb *VvADH1* promoter analysis revealed no ERE motifs, although two ARE-like GT-motifs were identified. However, these motifs were not sufficient to confer inducibility by anaerobiosis to the *VvADH1* promoter (Torregrosa et al. 2002). ERE and ARE motifs were also identified in promoters from *Arabidopsis AtADH1* and tomato

LeADH2 genes (Fig. 4). Compared to that of *VvADH2*, they are organized in a different way, especially when considering the proximal 300 bp promoter region. At the expression level, both *AtADH1* and *LeADH2* genes are transcriptionally induced by low oxygen stress (Chen and Chase, 1993; Dolferus et al., 1994). In addition, functional analysis showed that GT-motifs in the *Arabidopsis AtADH1* are critical for low oxygen induction (Hoeren et al., 1998). However, whether ERE motifs are involved in the transcription of these *ADH* genes remains to be established.

Discussion

Alcohol dehydrogenase has received considerable attention as a stress marker, its expression being induced by several environmental factors, such as anaerobiosis, drought, chemical treatment or low temperature (Kadowaki et al., 1988; Matton et al., 1990; Millar et al., 1994; Christie et al., 1991), and hormones (de Bruxelles et al., 1996; Lu et al., 1996, Peng et al., 2001). The analysis of the mechanisms underlying this genetic control has revealed interaction between different signalling pathways in the *Arabidopsis ADH1* promoter (Dolferus et al., 1994).

In this work, treatment with 1-MCP has been shown to result in a noticeable decrease of ADH activity and *VvADH2* mRNA expression in ripening berries. Similar results showing the inhibition by 1-MCP and the induction by CEPA-generated ethylene were observed when enzyme activity and transcripts were analysed in cell suspensions. The 1-MCP effect on fruits is obvious only after veraison has been initiated in a majority of grape berries. This fits with previous reports showing that grape berries respond to ethylene differently according to the time elapsed since flowering (Hale et al., 1970), although a low level of ethylene is expected to be produced at the time of ripening (Coombe and Hale, 1973). The effect achieved here with the use of both chemical ethylene release and anoxia treatments indicates that both signals participate in the control of *VvADH2* transcription. If ethylene *per se* was directly involved in the induction of grapevine *ADH* expression, one would expect an increase in ethylene content in ripening berries or in hypoxically-treated cells. The absence of a significant release of ethylene in ripening berries (Coombe and Hale, 1973) or in suspension cells with increased ADH suggests that this expression was not directly under ethylene control. It is rather likely that ethylene and low oxygen use different signalling

pathways. However, it is not excluded that low oxygen may sensitise the *VvADH2* promoter to ethylene action.

The fact that *VvADH2* transcription is partially reduced after veraison by the 1-MCP treatment indicates that the *ADH* gene is at least in part responsive to ethylene. But we can not exclude an indirect action of MCP on ADH expression (i.e. regulation of intermediate metabolisms). Moreover, the stronger effect observed with repeated MCP treatment after veraison - although single and repeated treatments did not differ before this stage - suggest some changes in the regulation of the ethylene signalling pathway during fruit development. Functionnal receptors are a first requisite for the downstream response of genes related to this pathway. Whether the regulation occurs at the level of ethylene receptors (e.g. variation in the number of functional receptors or competition at the receptor level) and / or downstream in the transduction pathway remains an open question. Ongoing studies on ethylene in grapes (Chervin et al., 2004) led the authors to consider it as an important signalling pathway for grape berry ripening. Indeed, the ethylene production peaks two weeks before mid-veraison, i.e. just before the onset of *VvADH2* expression (Fig. 2A). In addition, 1-MCP applied at the time of the ethylene peak has been shown to alter several events (e.g. increase in berry diameter and anthocyanin accumulation) involved in berry ripening (Chervin et al., 2004). Moreover, in tomatoes, the expression of some receptors are triggered at the inception of ripening (Ciardi and Klee, 2001). Another possibility is that some ethylene biosynthesis genes, or genes involved in the signalling pathway, are activated. It could be that *ADH* first has to be induced *via* a limitation of oxygen (Martinez et al., 1993), before ethylene signalling becomes active (as a consequence or not of lesser oxygen availability). Microarray analysis of roots from *Arabidopsis* responding to low oxygen treatment have shown that ethylene biosynthesis and response were induced later on in the low oxygen response, while *ADH* expression was already reaching maximal RNA levels after 4 h (Klok et al., 2002). The ethylene response could therefore be considered as a consequence of low oxygen treatment. Moreover, in *Arabidopsis*, mutations affecting ethylene responses (and in particular receptor of ethylene) also affected the *ADH* induction, also at later stages of hypoxia (Peng et al., 2001).

This report shows that, in grapevine, ethylene signalling was more efficient in increasing ADH transcription when associated with low oxygen treatment. In fact,

ethylene appears to be implicated in the triggering of a number of responses to oxygen deficiency (Morgan and Drew, 1997). Because the *VvADH2* promoter has putative ERE motifs, the expression of this gene could indeed be sustained or amplified by ethylene. In fact, recent experiments have shown that the promoter of the *VvADH2* gene was responsive to ethylene treatment (Verries et al., 2004). Finally, it is not excluded that some other transcription factors are required in the fruit ripening processes. In this case, both anaerobic and ethylene pathways would be dependent on one or more transcription factors.

Results presented here suggest that the combination of both signals could be involved in *ADH* expression in fruit tissues. Whether anaerobic conditions can be encountered in some grape berry cell compartments remains to be shown. It is however becoming evident that anaerobic metabolism in plants is not always activated by a decrease in oxygen availability. Anaerobic metabolism may also function as a mechanism to reduce energy consumption under certain circumstances (Geigenberger, 2003).

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Table 1. *Ethylene concentrations (ppm) measured in the headspace above V. vinifera (cv Cabernet Sauvignon) suspension cells. Cells from 5 days old suspensions were treated as indicated in Material and Methods with gaseous 1-MCP (1 ppm) for 2 h (MCP), and / or gazeous CEPA (50 µM) for another 2 h (respectively CEPA and MCP+CEPA). Untreated cells were used as control. Cells were maintained for 24 h either in air or under anoxia.*

Means of two independent experiments ± standard errors.

C_2H_4 (ppm)	Start	24 h air	24 h N_2
Control	<0.04	<0.04	<0.04
MCP	<0.04	<0.04	0.06 ± 0.03
CEPA	1.70 ± 0.40	4.25 ± 0.15	1.05 ± 0.65
MCP + CEPA	2.70 ± 0.90	2.45 ± 0.45	1.05 ± 0.65

Legends

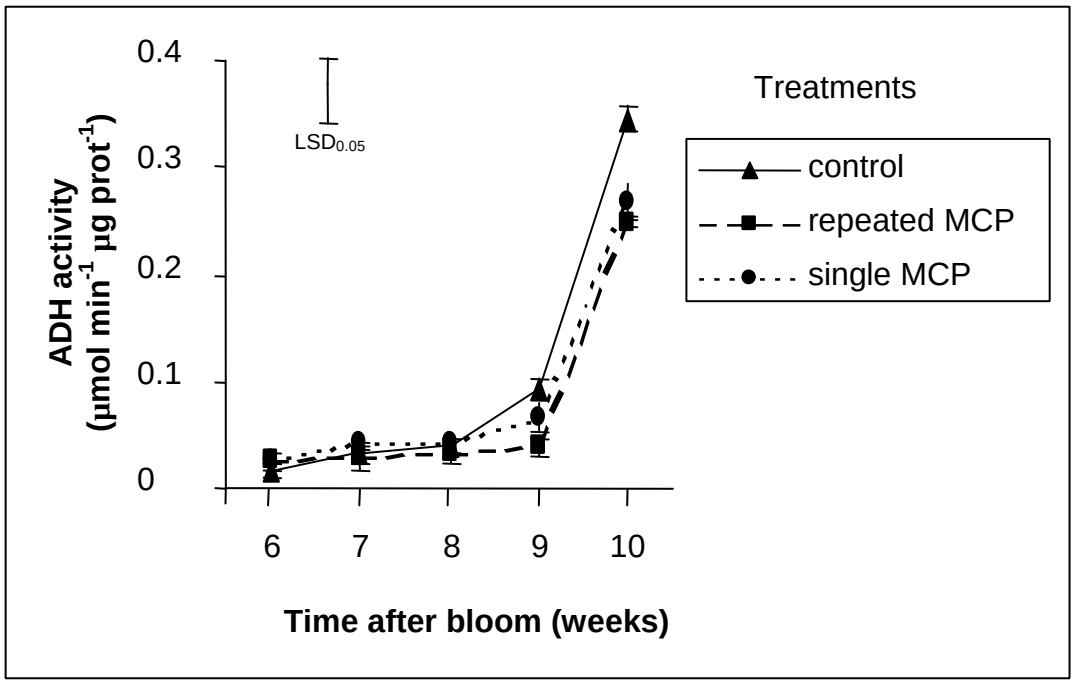
Fig. 1. Specific ADH activity in Cabernet Sauvignon berries treated with MCP at different stages of fruit development. Veraison initiated at week 8. Error bars indicate the standard deviation (n = 2). The LSD bar shown in the top left corner represents the significant difference at the 0.05 level for the treatments-time interaction.

Fig. 2. Accumulation of *VvADH2* mRNAs in Cabernet Sauvignon berries, treated with MCP at different stages of fruit development, using different application regimes (repeated or single). Veraison initiated at week 8. (A) Northern blots were probed with specific *VvADH2* 3' UTR probes (UTR = untranslated region). The signals were quantified and normalized in each lane to the corresponding ribosomal 18S signal. Error bars indicate the standard deviation (n = 3). The LSD bar shown in the top left corner represents the significant difference at the 0.05 level for the treatment-time interaction. (B) Effect of MCP on *VvADH2* mRNA expression levels. Expression levels are expressed as percentage of control expression levels.

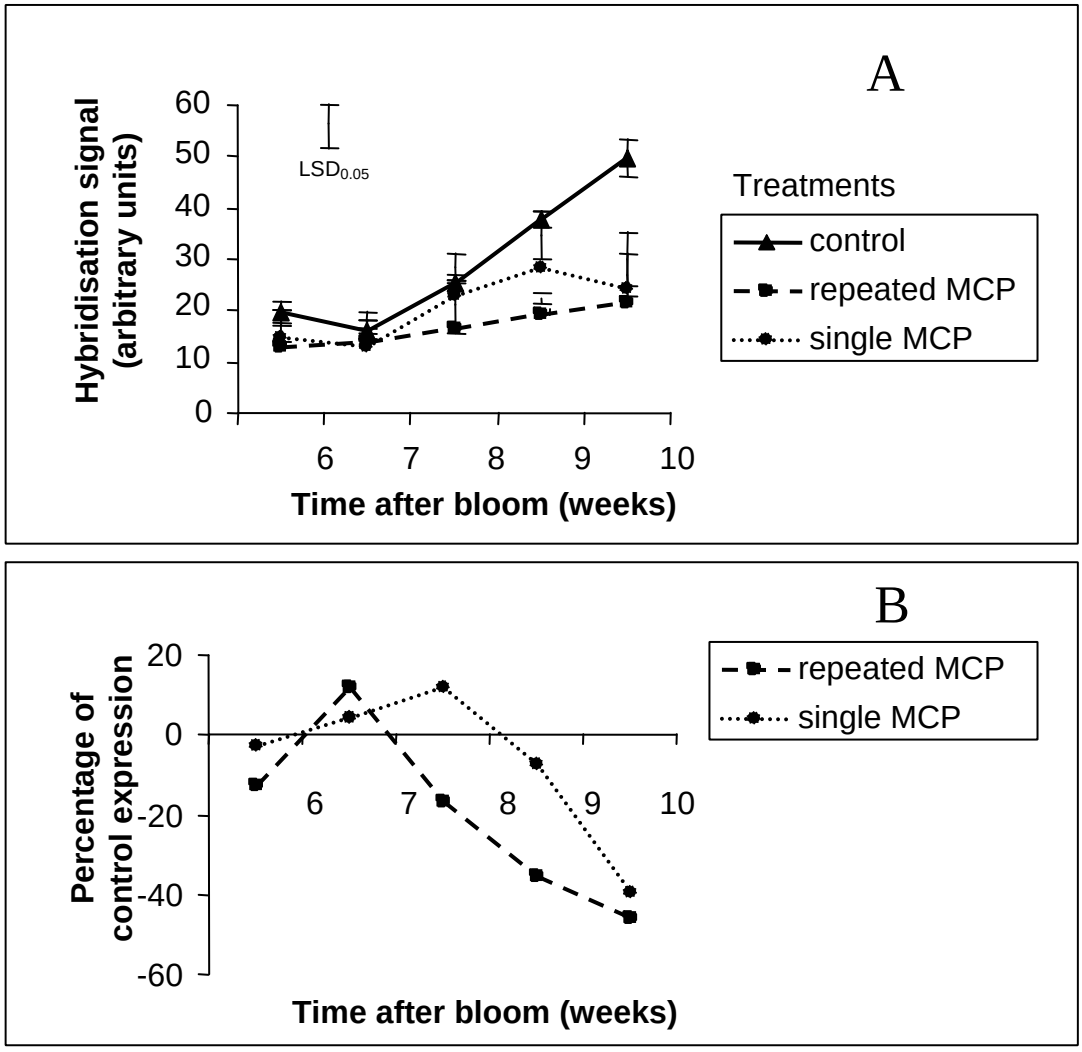
Fig. 3. (A) Relative ADH activity and (B) *VvADH2* relative mRNA expression level in *V. vinifera* L. cell suspensions (cv. Cabernet Sauvignon) as a function of various treatments and incubation under air or nitrogen in the following 24 h. Northern blots were hybridised first with a *VvADH2* 3' UTR specific probe (UTR = untranslated region), then with a 18S rRNA probe for gel loading corrections. The resulting signals (arbitrary units) and the ADH activity levels were normalised taking a constant value of 1 for the corresponding controls at time 0. Cells from 4 days old suspensions were treated for 2 h with 1ppm gaseous 1-MCP (MCP), and / or gaseous CEPA (50 µM) for 2 additional hours (respectively CEPA and MCP+CEPA). Control corresponds to untreated cells. Error bars indicate the standard deviation (n = 2). The LSD bars shown in the top left corner represent the significant differences at the 0.05 level for the treatments-atmosphere interaction.

Fig. 4. Positions of ERE (ethylene-responsive-elements) and ARE (anaerobiosis-responsive-elements) putative cis-regulators within the 1 kb promoter region of different *ADH* genes. *Vv* stands for *V. vinifera*, *LE* for *Lycopersicon esculentum* (GB accession number X77233) and *At* for *Arabidopsis thaliana* (GB accession number M12196).

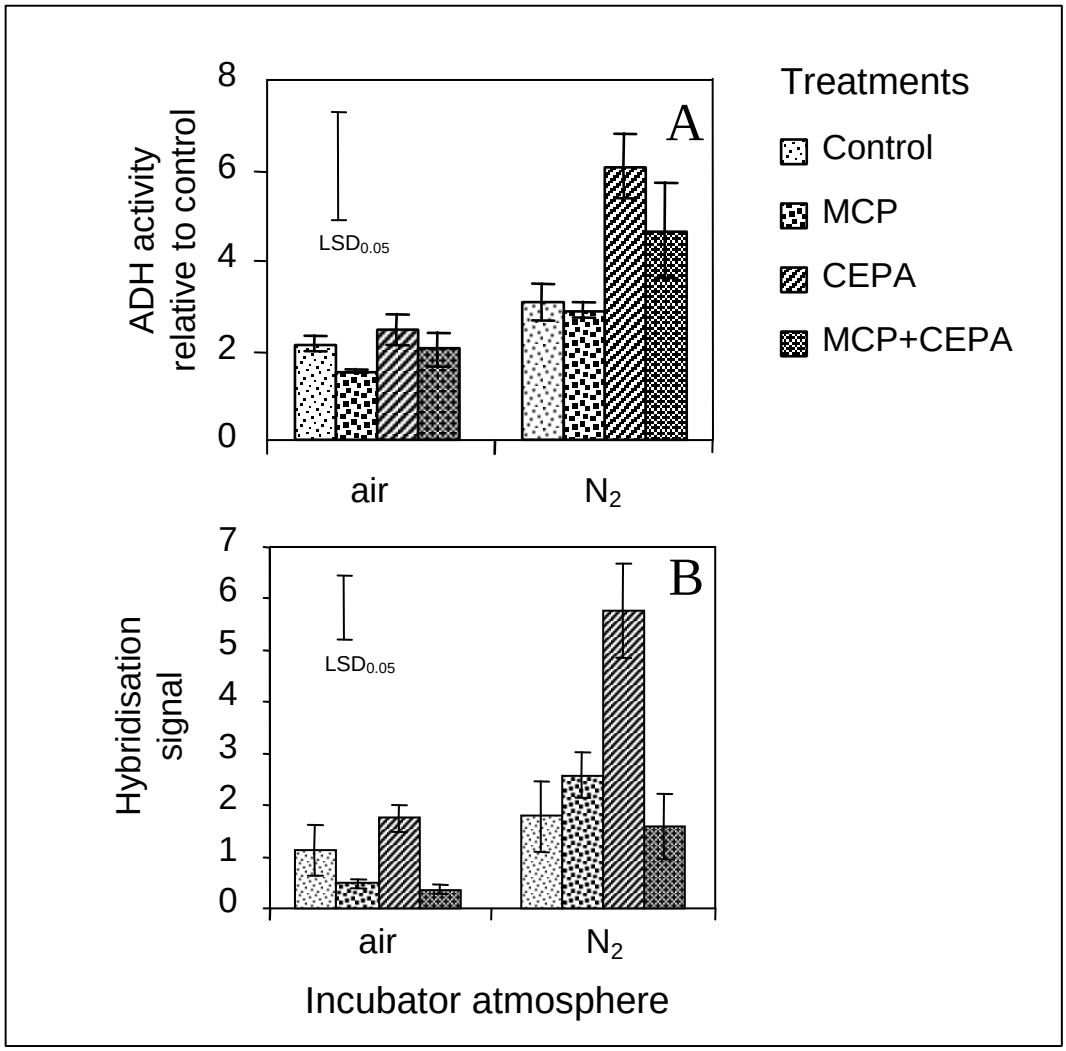
421 **Fig.1.**



422 **Fig. 2**



423 **Fig. 3.**



424 **Fig. 4.**