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Larrigaudiere C., Vendrell M.

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EFFECT OF COLD STORAGE ON ETHYLENE BIOSYNTHESIS CAPACITY IN GRANNY SMITH APPLES

Larrigaudiere, C
UdL-IRTA, Lleida, Spain

Vendrell, M.
CSIC, Centro de Investigacion y Desarrollo, Jorge Girona, Barcelona, Spain

Abstract

Changes in respiratory rate, 1-aminocyclopropane-1-carboxylic acid (ACC) metabolism and ripening in Granny Smith apples (*Malus domestica* Borkh L., cultivar "Granny Smith") were studied in relation to cold storage. Fruits were held at 4° C or 22° C for 31 days (4° C controls and 22° C controls, respectively) or at 4° C for periods of 2, 5 or 10 days before transfer to 22° C for the rest of the 31-day period. Ethylene and CO_2 were produced at a low rate in 22° C controls. In 4° controls, ACC levels increased greatly, showing an inhibition of both ethylene-forming enzyme (EFE) and malonyl trasferase activities. in chilled fruit, ethylene, malonyl ACC (MACC) levels and EFE activity increased sharply after transfer to 22° C. These increases were accompanied by a significant rise in the respiratory rate, similar to the activation of a climacteric process. Chilled fruit also showed a loss of pulp firmness and acidity compared with fruit kept continuously at 22° C.

The effect on ethylene biosynthesis in fruit harvested at different stages of maturity and stored at 1° C for a short period (10 days) was also studied. Cold is a strong inducer of ethylene biosynthesis in less mature fruit. After transfer of the fruit to room temperature (20° C), there is a tendency to uniformity of ethylene production an ripening in fruit harvested at different times. This effect seems to be a consequence of an activation of the enzyme ACC oxidase as shown above, which is the limiting factor of the capacity of the fruit to produce ethylene, and is age dependent.

Keywords

CLIMACTERIC BEHAVIOR, COLD STRESS, ETHYLENE, "GRANNY SMITH" APPLESABBREVIATIONS: ACC = 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID; IRGA = INFRARED GAS ANALYZER; MACC=MALONYL ACC; SSC = SOLUBLE SOLIDS CONCENTRATION

1. INTRODUCTION

Although cold storage is the most usual means of delaying post-harvest deterioration in fruits and vegetables, refrigeration does not suppress all physiological changes. In some plant species such as cucumbers (Wang and Adams, 1982) and Phaseolus vulgaris leaves (Field, 1981), refrigeration can induce ethylene production. Such temperature-induced changes in ethylene production may have a

significant impact on plant growth and development (Field and Barrowclough, 1989). This is particularly evident for some climacteric fruits such as Bosc pears (Sfakiotakis and Dilley, 1974) and d'Anjou pears (Blakenship and Richardson, 1985), which require exposure to cold temperature for ripening to occur.

The increase in ethylene production in pears was found to be a result of an increased capacity to produce 1-aminocyclo-propane-1carboxylic acid (ACC) (Knee *et al.*, 19830. However, ACC synthase activity remains generally low in cold conditions and increases only after transfer to a higher temperature (Knee, 1987). The activation is inhibited by cycloheximide but not by cordycepin (Wang and Adams, 1982), showing that, in contrast to the transcription process which can be stimulated during cold storage, the translation process was not completed until transfer to warmer conditions. Concomitantly, the system converting ACC to ethylene (ethylene-forming enzyme (EFE) or ACC oxidase) is inhibited by cold treatment (Wang and Adams, 1980). After transfer to higher temperature, recovery is possible but it is inversely related to the period of cold storage (Etani and Yoshida, 1987).

The effect of cold on the development of the ethylene biosynthetic pathway in pears is of interest, since a regulatory influence of temperature on the onset of ethylene production could provide a good model with which to study the induction of the climacteric process. This is also true for all other climacteric fruits which act similarly, and particularly for the apple cultivar "Granny Smith".

Here, we extend the study of the effect of cold on the ethylene biosynthetic pathway of this fruit as reported by Jobling *et al.* (1991), especially in relation to the ACC oxidase activity and its consequences on the ripening behavior. Since the maturity stage can be an important factor on the behavior of the fruit, the effect of cold temperature on fruit harvested at different stages of maturity, near the optimal commercial harvest date, was also studied.

2. MATERIALS AND METHODS

Plant material and storage conditions

The apples (*Malus domestica* Borkh L.cv. "Granny Smith") were obtained from Lleida and harvested at commercial maturity (202 days after full bloom). The apples were stored in a chamber at a relative humidity of about 85% and a temperature of 4° C or 22° C for 31 days (4° C controls and 22° C controls respectively), or at 4° C for periods of 2,5 or 10 days before transfer to 22° C for the remainder of the experimental period.

For the study of the effect of age, fruit was harvested on September 20 (M1), October 5(M2) and October 19 (M3). After harvest, the fruits were divided in three sets. One was kept at room temperature (20° C) another was stored at 1° C for 10 days and then transferred at 20° C.

Determination of ethylene, CO2 production, ACC and malonyl ACC levels

Individual fruits were placed in 350 ml flasks continuously aerated with humidified air. The flow rate was about 1 l h^{-1}. At various times, 1 ml gas samples were taken and injected into a gas chromatograph for ethylene determination. CO_2 concentrations were measured by connecting the effluent air from the ventilated flasks to an infrared gas analyser (IRGA) (COSMA, diamant 6000).

For determination of ACC, samples were extracted with 80% ethanol and the ACC content was assayed according to the method of Lizada and Yang (1979). Malonyl ACC (MACC) was measured by analyzing the ACC content of an extract hydrolised as described by Hoffman *et al.* (1982). Yields were routinely about 85%.

ACC oxidase activity

ACC oxidase activity was determined *in vivo* by measuring the conversion of administered ACC to ethylene.

Cylinders of pulp tissue removed radially with an 8 mm steel cork borer were cut into discs uniformly 1 mm thick. Six discs of pulp tissue from each fruit were incubated in a 20 ml vial containing 3 ml of incubation medium (0.4 M mannitol, 50 mM MES-KOH, pH 7.0) in the presence of 5 mM ACC. Sealed flasks were then incubated at 22° C for 3 h and gas samples were taken for C_2H_4 analysis by gas chromatography. All these operations were carried out at 4° C for the cool control. The wound ethylene production induced by preparation of the discs was observed only after 8 h (result not shown) and did not affect our measurements.

Measurement of firmness

Fruit firmness of all samples was estimated after the 31 day storage period. Firmness was measured on two opposite peeled side of four fruits samples using a penetrometer fitted with an 8 mm diameter probe.

Soluble solids concentration (SSC)

Soluble solids concentration (SSC) was determined by measuring the refractive index of the juice of the same fruits used for firmness determination. The data represent the means of two opposite pulp sides of four fruits and are expressed as a percentage (g per 100 g fresh weight).

Measurement of acidity

Acidity was measured as follows: 10 ml of pulp juice were diluted with 10 ml H_20 and titrated against 0.1 N NaOH solution. The acidity was expressed in g of malic acid per litre of juice.

3. RESULTS

Cold-induced climacteric rise in C2H2 and CO2 production.

In 22° C control fruit, ethylene production remained low. After 15 days, it increased slightly, then levelled off at a constant level of 7 nl g^{-1} h^{-1} (Fig. 1). After 10 days, 4° C control fruit showed a slight increase in ethylene production. thereafter this

remained constant at 2 nl g^{-1} h^{-1} . A sharp increase in ethylene production was demonstrated in 2-, 5- and 10 days chilled fruit following transfer to the higher temperature. This increase was immediate for 10-day chilled fruit but was only detected 4 days after transfer in the fruits held for 5 days at 4° C. In both cases, the maximum of ethylene production was about the same (23.4 and 23.7 $^{-1}$ h^{-1} respectively). Cold activation of ethylene production was also observed in 2-day chilled fruit. However, in this case, the period between transfer to 22° C and the increase in ethylene production was greater (17 days) and the maximum of ethylene production was lower (17.5nl g^{-1} h^{-1}).

Control fruit held continuously at 4° C produced low (3 ml kg $^{-1}$ h $^{-1}$) but constant levels of CO_2 (Fig. 20. The rate of respiration of 22° C control fruit was greater (about 9 ml kg^{-1} h^{-1}) and less stable , and it increased only when ethylene production was higher. In fruits kept initially at 4° C then transferred to 22° C, CO_2 levels increased sharply after transfer to the higher temperature. This increase was rapid (cf. Fig. 2 (insert). Maxima in CO_2 production were similar (24.6 and 27.3 ml kg $^{-1}$ h $^{-1}$) for 10-day and 5-day chilled fruit respectively) and peaked at the same time (14 days after transfer). Thereafter, CO_2 levels decreased and levelled off at a value twice that of 22o C control fruit. For 2-day chilled fruit, the CO_2 increase was also significant but biphasic and with smaller peaks. The second (larger) peak was observed 19 days after transfer.

Changes in ACC and MACC levels

Fig. 3 shows the results for pulp tissue. In 22^0 C control fruit, ACC increased slightly after 15 days, when ethylene production was initiated. In contrast, in 4^0 C control fruit, ACC levels increased after about 7 days. Following transfer from 4^0 C to 22^0 C, and in spite of a significant increase in ethylene production (Fig. 10, ACC levels in 5-day chilled fruit remained at a constant, low level. In 10-day chilled fruit, the ACC level decreased upon transfer to the higher temperature, and stabilized 3 days later at a level similar to that of 22^0 C control and 5-day chilled fruit.

In spite of the high ACC levels, 4^0 C control fruit contained only low amounts of MACC. In 22^0 C control fruit, MACC levels were low until day 15 of storage, when they began to increase. Five- and 10-day chilled fruit showed a significant increase in MACC levels following transfer. The timing of these changes was as previously described for C_2H_4 production, being immediate for 10-day chilled fruits, but visible only after 5 days following transfer for 5-day chilled fruit

Effect on ACC oxidase activity

In both 4^0 C and 22^0 C control fruit, ACC oxidase activity was low (Fig. 4), increasing in the latter after 15 days, when ethylene production was significant. In contrast, chilled fruit showed a sharp increase in ACC oxidase activity after transfer. Once more, the timing of the increase was different for 10-day and 5-day chilled fruits, as described above.

Consequences for fruit quality

Three parameters were studied: firmness, acidity and the refractive index of the pulp. Fruit held for 10 days at 4^0 C before transfer to 22^0 C showed an important loss of pulp firmness and acidity compared with 22^0 C control fruit (Table 1). Changes in sugar content were not significant.

Effect of maturity stage on cold-induced ethylene biosynthesis

At $2O^0$ C, ethylene production after harvest depend on the maturity stage of the fruit (Fig. 5A). It was negligible in less mature fruit (M1) for several weeks. In more advance fruit, ethylene production began earlier and this advancement was related to maturity (M2 and M3) the effect of cold treatment (10 days at 1^0 C) was marked. After transfer to normal room temperature (20^0 C), fruits picket at any stage of maturity showed a similar high increase in ethylene production (Fig. 5B). ACC oxidase activity parallels ethylene production in relation to the development of the fruit and to the effect of cold treatment (Fig. 6A and B).

4. DISCUSSION

The "Granny Smith" cultivar shows a typical ripening behavior. At 22° C, it ripens slowly, producing little ethylene and respiring slowly. Furthermore, although classified as a chilling-resistant fruit, it is in fact sensitive, producing high levels of ACC at 4° C (Chen and Patterson, 19815) This increase in ACC levels may have been due to two enzymatic inhibitions which occurred at 4^0 C . The first concerns ACC oxidase activity, probably as a result of the cold-induced alterations of membrane properties (Steponkus, 1984).However, in view of the recent characterization of ACC oxidase as a soluble enzyme (Ververidis and John, 1991; Dupille *et al.*, 1992) it seems unlikely that the membrane affects this enzyme directly. Its action is probably indirect and may involve a control of transmembrane gradients and/or thermodynamic regulation of the enzyme activity. The second concerns the inhibition of malonyl transferase. Indeed, in spite of high ACC levels, MACC did not increase at 4^0 C. This inhibition may not be a consequence solely of thermodynamic events, metabolic inhibition could also be involved.

Considering the high levels of ACC at 4^0 C and the relative low activity of ACC oxidase and malonyl transferase at 4 and 22^0 C, it cannot be concluded that ACC accumulation was a consequence of these enzymatic inhibitions alone. Activation of ACC synthase may also be involved, which could be confirmed by *in vitro* analysis and molecular experiments.

After transfer from 4 to 22^0 C, and as recently reported by Jobling et al. (1991). "Granny Smith' apples showed a strong increase in both ethylene and CO_2 production. This increase in ethylene production was immediate for fruits chilled 10 days but retarded for fruits chilled for only 5 days. The reasons for this activation should be sought in a regulatory system: a dynamic process which probably involves the destruction of ripening inhibitors (Reid et al., 1973) or the activation of ripening-

related genes (Wilson et al., 1990). Activation of ACC synthase is a possible candidate. This activation occurs at 4^0 C and probably remains after transfer of the fruits to 22^0 C. However, this was not obvious from the determination of ACC alone which was largely converted to ethylene and MACC.

In agreement with Jobling et al. (1991), we can now confirm that the cold-induced activation of ethylene production involves activation of EFE. The physiological basis of this activation remains unclear. It may involve a "maturation" process equivalent to the system proposed by McMurchie et al. (1972) in which EFE would be transformed into a climacteric sensitive system.

Carbon dioxide levels also increased after fruits were transferred to the higher temperature. The maxima occurred at the same time (14 days after harvest) for both the 5-day and 10-day chilled fruit, showing a programmed process which may be associated with the climacteric crisis.

Activator of the ethylene biosynthetic pathway was not without consequences for the ripening-related qualitative attributes of apples. It brought a change in some ripening parameters and a loss of flesh firmness and acidity but no significant change in sugar content. Such stability of the sugar level during ripening has been confirmed in other experiments.

While ethylene production of harvested fruit depends on the maturity stage, cold treatment produces a homogeneous ethylene production capacity after transfer to room temperature. ACC oxidase appears to be crucial to this. During cold conservation, this enzyme accumulates (Lelievre et al., in press). Immediately after transfer at 20^0 C, the activity increases and is responsible for the high ethylene production (Larrigaudiere and Vendrell, 1993). In all cases the correlation between ethylene production and ASS oxidase activity is clear. The activation of this enzyme is more evident in less mature fruits. These data indicate that cold is an activator of ethylene biosynthesis in "Granny Smith" apples.

The role of ACC in the induction of ethylene production seems to be less important. There is no indication that this substrate is a limiting factor. There was a marked amount of ACC in all samples (data not shown) and these levels also increased after transfer to room temperature and were similar in all cases. In any case, this means an activation effect of cold treatment on ACC metabolism.

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LEGENDS TO FIGURES

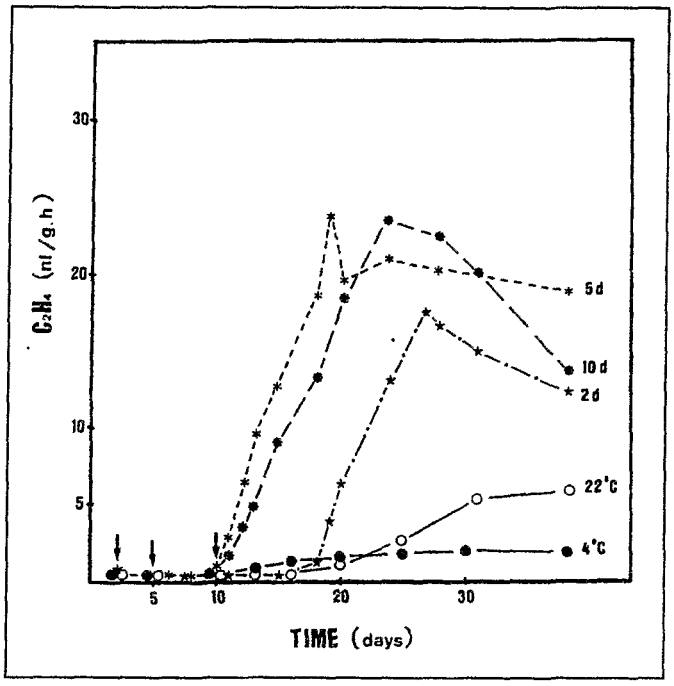

Fig. 1. Effect of cold storage on the rate of ethylene production in "Granny Smith" apples. Arrows indicate the times of transfer to the higher temperature. Values represent means of four replicates +/-25%.

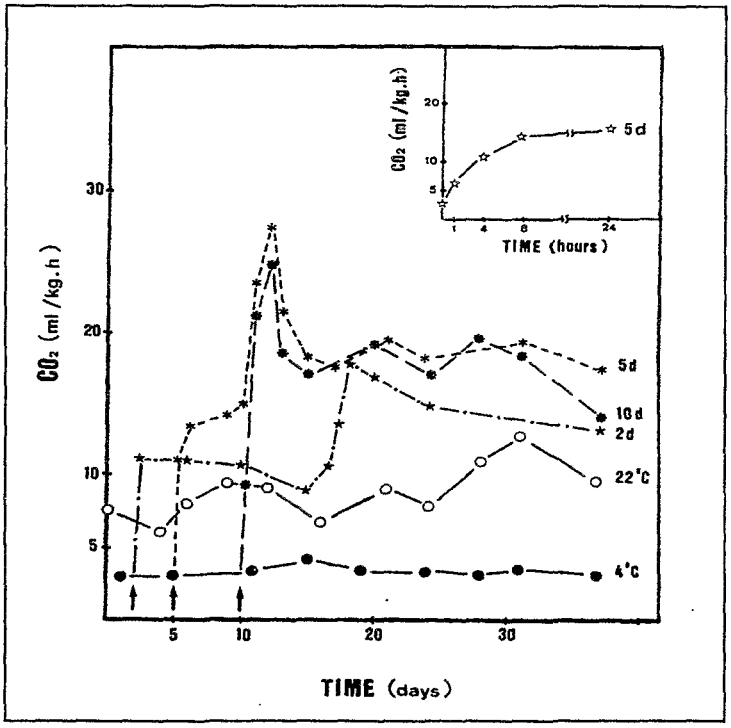

Fig. 2. Effect of cold storage on the respiratory rate of "Granny Smith" apples at 22° C. Arrows indicate the times of transfer to the higher temperature. Values represent means of four replicates +/-25%.

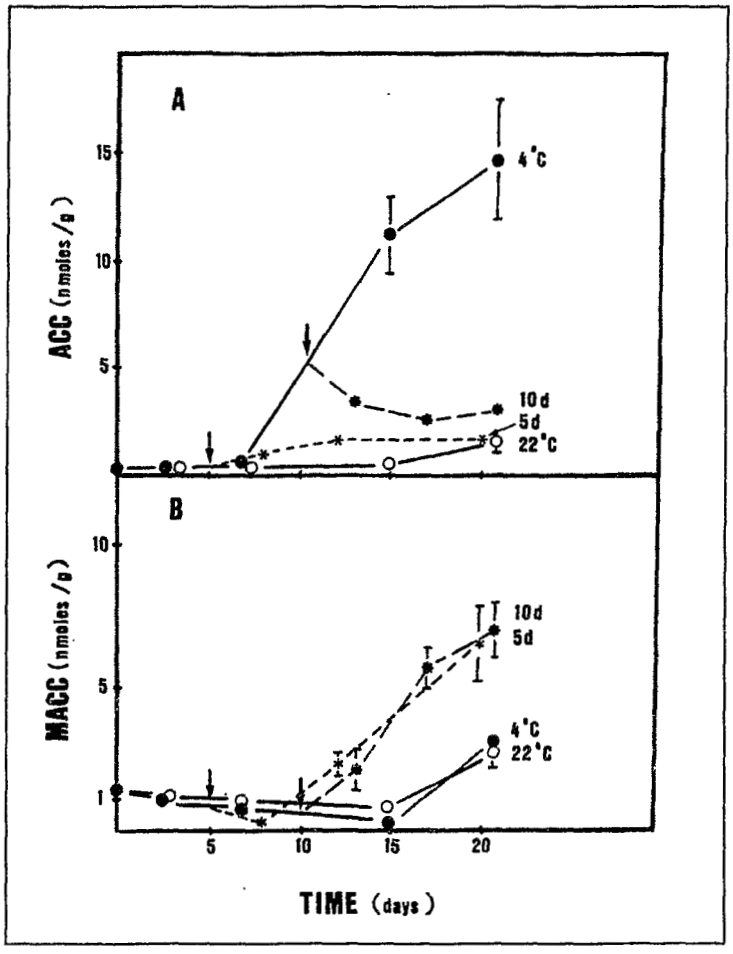

Fig. 3. Changes in the levels of ACC (A) and MACC (B) in pulp tissue of "Granny Smith" apples after transfer to 22°C. Arrows indicate the times of transfer to the higher temperature. Values represent means of three replicates +/-S.D.

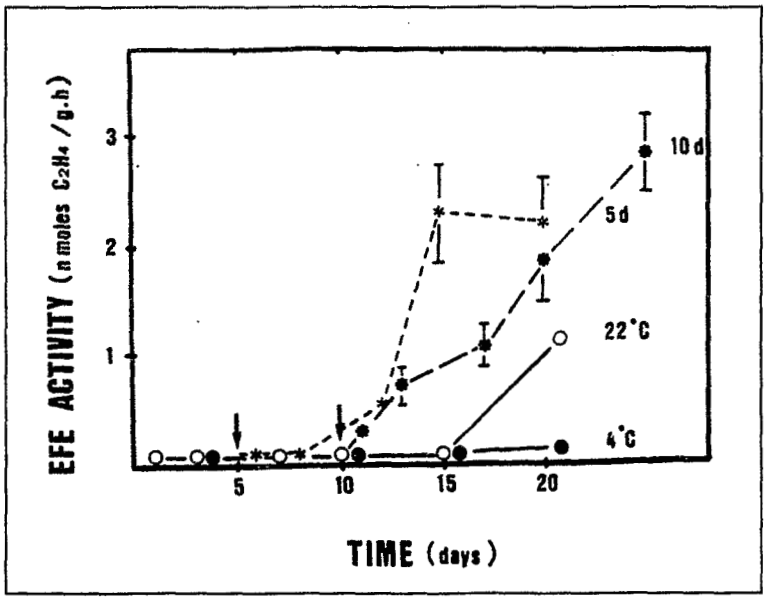

Fig. 4. Cold-induced changes in ACC-oxidase activity in pulp tissue of "Granny Smith" apples after transfer to 22° C. Arrows indicate the times of transfer to the higher temperature. Values represent means of three replicates +/-S.D.

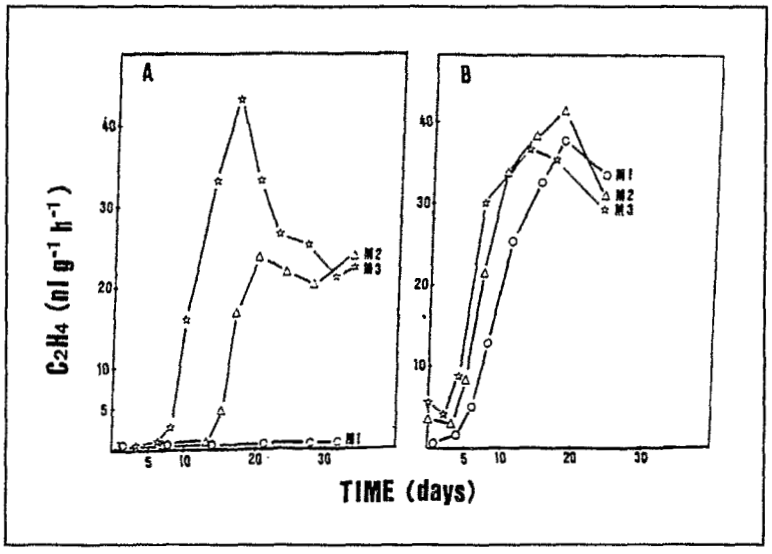

Fig. 5. Effect of cold treatment (10 days at 1° C) on ethylene production in Granny Smith apples harvested at different stages of maturity (M1: 20/09, M2: 05/10, M3; 19/10). A): Fruit kept at 20° C, B): Apples stored at 1° C for 10 days and then transferred at 20° C (time).

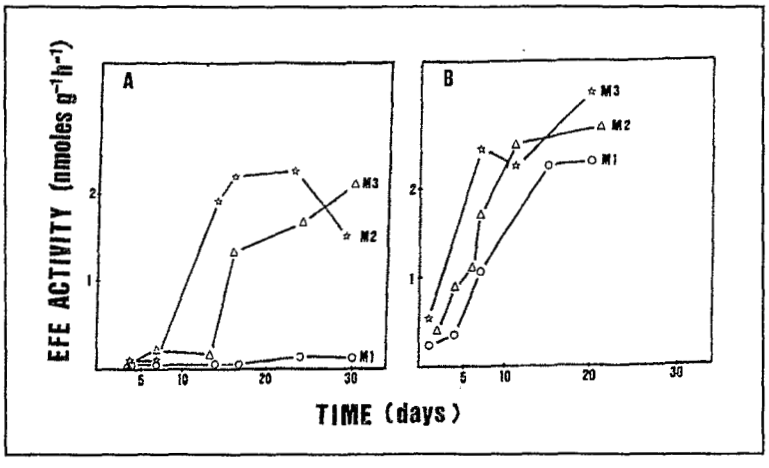

Fig. 6. Effect of cold treatment (10 days at 1° C) on ACC oxidase activity in Granny Smith apples harvested at different stages of maturity (M1: 20/09, M2: 05/10, M3; 19/10). A): Fruit kept at 20° C, B): Apples stored at 1° C for 10 days and then transferred at 20° C (time).

Table 1. Effect of cold treatment on qualitative parameters of "Granny Smith" apples: pulp firmness, refractive index (R.I.) and acidity. Control fruits were held for 31 days at 22° C. Cold-treated fruits were kept at 4° C for 10 days and then transferred to 22° C for 21 days for further ripening. Values represent means of four replicates ± S.D.

	Firmness (kg cm^{-2})	RI (%)	Acidity (g malic acid l^{-1})
Control fruit (22° C)	7.6 ± 0.6	11.9 ± 0.3	6.4 ± 0.3
Treated fruit (10 days at 4° C)	5.7 ± 0.3	12.0 ± 0.5	5.5 ± 0.1