

# Delayed storage and controlled atmosphere storage of nectarines: two strategies to prevent woolliness

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Received 21 May 1999; accepted 11 October 1999

## Abstract

Both delayed storage (DS) of nectarine (*Prunus persica* cv. Flavortop) fruits held for 48 h at 20°C before storage, and controlled atmosphere (CA) storage, fruits stored at 10% CO<sub>2</sub>, 3% O<sub>2</sub>, alleviated or prevented chilling injury manifested as woolliness in nectarine fruits stored for 4 or 6 weeks at 0°C. Control fruits showed 80 and 100% woolliness during ripening after 4 or 6 weeks at 0°C, respectively. DS and CA were similar in their beneficial effect after 4 weeks and CA was better after 6 weeks storage. The two storage processes appeared to prevent woolliness by different mechanisms. DS initiated ripening so that at removal from storage polygalacturonase (PG) activity was higher and pectin esterase (PE) activity lower than in control fruits. The PG activity increased further during ripening, and normal softening occurred in DS fruits. There was no difference in mRNA abundance of PG and PE between DS and control fruits. CA repressed both mRNA levels and activity of PG during storage, but allowed recovery of activity during ripening. Endoglucanase (EGase) activity declined during ripening in all fruits, but control fruits retained more activity than DS or CA fruits. The EGase mRNA level was high in control fruits during ripening after storage, and almost undetectable in all treatments at all other times. We postulate that the ratio between PG/PE either at removal (DS) or during ripening (CA) will determine whether woolliness develops or not. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Nectarine; Delayed cooling; Controlled atmosphere; Woolliness; Polygalacturonase; Pectin esterase; Endoglucanase

## 1. Introduction

Most nectarine cultivars develop chilling injury (CI) if they are held for more than 2–3 weeks below 8°C (Lill et al., 1989). The injured fruits

have a good appearance when removed from storage, but do not ripen normally. The main symptom of CI is woolliness of the flesh, leading to a dry, mealy texture when eaten. Since this disorder is not apparent externally, fruits are often marketed at this stage, which can lead to decreased consumer acceptance.

The problem of mealiness or woolliness had been recognized for a long time in peaches and

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nectarines, and strategies have been developed to prevent its onset by regimes of intermittent warming (IW), with or without controlled atmosphere (CA), CA alone, or delayed storage (DS) (Ben-Arie et al., 1970; Anderson and Penney, 1975; Ben-Arie and Sonogo, 1980; Wade, 1981; Anderson, 1982). However, IW alone or combined with CA is cumbersome during long distance shipping or in large storage rooms. DS has produced inconsistent results, alleviating the onset of woolliness in some studies, but ineffectiveness has also been reported, accompanied by greater weight loss and fruit softening without reducing internal breakdown (Wade, 1981; Tonini et al., 1989).

IW and DS are postulated to promote the dispersal of potentially phytotoxic volatile compounds (Bramlage, 1982). However, more attention has been concentrated on the effects of these treatments on cell wall metabolism (Dawson et al., 1992, 1995; Lurie et al., 1994). Ben-Arie and Sonogo (1980) found that there was an imbalance between polygalacturonase (PG) and pectin esterase (PE) activity during woolliness development by comparing IW treatment with fruits placed directly into storage. With relatively high PE and low PG activities in chilling-injured fruit, the pectin matrix is de-esterified, without subsequent depolymerization, leading to large pectin molecules with low esterification. This kind of pectin is assumed to form a gel, possibly aided by calcium in the cell wall, which will bind free water and cause woolliness symptoms.

CA with elevated CO<sub>2</sub> and reduced O<sub>2</sub> concentrations was found to delay or prevent woolliness (Lill et al., 1989). Cell wall changes following CA storage appeared to be similar to changes during ripening without storage, where woolliness did not develop (Lurie et al., 1994). However, cell wall enzyme activities were not examined in these fruit, nor the presence and abundance of mRNAs of the enzymes. The present study was designed to compare the effects of DS and CA on preventing woolliness in 'Flavortop' nectarines, and to see if prevention is related to the activity of hydrolytic cell wall enzymes.

## 2. Materials and methods

Nectarine (*Prunus persica* cv. Flavortop) fruits were harvested at commercial maturity according to fruit size and skin background colour (green-yellow). Fruits were divided into three lots. One lot was stored immediately in 0°C, 95% RH air (control), the second placed at 20°C, 80% RH for 48 h before transfer to 0°C air (delayed storage (DS)), the third was stored at 0°C, 95% RH in 40 l chambers supplied with a flow of 10% CO<sub>2</sub> and 3% O<sub>2</sub> once the fruit temperature reached 0°C (CA). This CA formula had been shown previously to be the best one for Israeli nectarine cultivars (Lurie, 1992).

Fruits were removed from cold storage after 4 and 6 weeks and subsequently held at 20°C for 7 days for ripening. On the day of removal and on the 3rd, 5th and 7th days of ripening, three replicates of ten fruits were sampled from each treatment.

Firmness was measured on two pared sides of each fruit using a penetrometer fitted with an 11-mm diameter plunger. After firmness was determined, the fruit was cut into two halves and woolliness was estimated both by visual observation and organoleptically. Juicy fruits with no signs of woolliness were classed as healthy.

The amount of expressible juice was determined by removing a tissue plug weighing about 2 g from each fruit with a cork borer, passing it through a 5 ml syringe into an Eppendorf tube, centrifuging and separately weighing the juice and solids (Lill and Ven Der Mespel, 1988). From each treatment, 30 extra fruits were weighed individually at harvest and during ripening to determine weight loss.

A wedge-shaped slice (approximate 5 g) was removed from each fruit in the replicates and the pooled sample was passed through an electric juicer (Moulinex, type 753 France) for the measurement of soluble solids content (SSC) and titratable acidity (TA) by refractometer and titration, respectively. A second batch of slices (45 g) was collected from each replicate and weighed before and after lyophilization for determination of tissue water content.

Two more samples (about 100 g each) were pooled, frozen in liquid nitrogen and held either at  $-20^{\circ}\text{C}$  for analysis of polygalacturonase (PG), pectin esterase (PE), and endo-glucanase (EGase) activities, or at  $-80^{\circ}\text{C}$  for mRNA preparation and analysis.

For enzyme extraction, 45 g of frozen fruit mesocarp were ground in 85 ml cold 12% PEG 4000, 0.2% Na bisulfite for 2 min. After centrifugation at  $10\,5000 \times g$  for 10 min the pellet was collected and separated into three parts for extraction of each enzyme activity. For exo-PG and endo-PG the pellet was incubated on a shaker at  $4^{\circ}\text{C}$  for 1 h in cold 50 mM Na acetate buffer pH 5, 0.5 M NaCl. Following centrifugation as above, the supernatant was diluted once with 50 mM Na acetate buffer, pH 5, and used as crude extract. Exo-PG activity was determined according to Gross (1982). Enzyme extract (0.15 ml) was mixed with an equal volume of 0.5% polygalacturonic acid in 50 mM Na acetate buffer, pH 4.4, and incubated at  $30^{\circ}\text{C}$ . Incubation was performed initially for 2, 4, 6, and 18 h and found to be linear. For convenience, 18 h was the incubation time chosen. For determination of galacturonic acid released, 2 ml borate buffer (0.1 M, pH 9.0) and 0.3 ml 1% cyanoacetimide were added to the reaction mixture, boiled 10 min, cooled and read at 274 nm. Galacturonic acid was used as a standard, and controls of boiled extract were run. One activity unit was 1  $\mu\text{g}$  galacturonic acid released per mg protein per h. Endo-PG activity was measured in a Cannon–Fenske viscosimeter by mixing 3 ml enzyme extract with 4.5 ml 2% polygalacturonic acid in 50 mM Na acetate, pH 4.4. Initial viscosity was measured and after a further 18 h incubation at  $30^{\circ}\text{C}$ . One activity unit was the change in viscosity (in s) per unit protein per h.

For PE extraction the pellet was resuspended into 15 ml 7.5% NaCl, 0.75% EDTA (pH 6.5) and incubated at  $4^{\circ}\text{C}$  for 10 min. Following centrifugation as above, the supernatant was collected. Five ml crude extract was mixed with 20 ml 1% citrus pectin and titrated with 0.01 N NaOH to maintain pH 7.4, while incubating at  $30^{\circ}\text{C}$ . The reaction was found to be linear for 2 h, but normally the reaction was measured for 30 min.

One unit activity was calculated as 1 mM NaOH consumed per mg protein per h.

For EGase activity, the pellet was stirred in 15 ml 0.1 M citrate phosphate buffer pH 6.0, 1 M NaCl at  $4^{\circ}\text{C}$  for 1 h, then centrifuged as above. Using the supernatant as crude extract, enzyme activity was measured by change in viscosity as for endo-PG. The reaction mixture contained 6 ml 0.2% methyl cellulose (CMC) in citrate phosphate buffer and 3 ml of the supernatant. Protein content was determined according to Bradford (1976) using Bio-Rad reagent.

Total RNA was extracted from 1g of freeze-dried flesh by the method of Lopez-Gomez and Gomez-Lim (1992). RNA samples were separated on 1% agarose/formaldehyde gels and transferred to nitrocellulose filters. RNA blots were pre-hybridized and hybridized in formamide buffers at  $42^{\circ}\text{C}$  as described in Sambrook et al. (1989). The final wash of the RNA blots was done in  $0.5 \times \text{SSC}$ , 0.1% sodium dodecyl sulfate (SDS) at  $60^{\circ}\text{C}$ . Probes were labeled with  $^{32}\text{P}$  by the random priming method, and included peach cDNA of endo-PG, PE (gift of Drs Diane Lester and Jim Speirs, Lester et al., 1994) and EGase (gift of Dr Pietro Tonutti, Bonghi et al., 1998). To measure loading variations, blots were re-hybridized with the 25 S ribosomal RNA (rRNA) gene probe (gift of Dr Mihaly Kis).

### 3. Results and discussion

#### 3.1. Alleviating woolliness by DS and CA storage

Both DS and CA storage markedly reduced woolliness during 7 days ripening after 4 and 6 weeks storage (Fig. 1(a,b)). Following CA storage with 10%  $\text{CO}_2$  and 3%  $\text{O}_2$  more than 90% of the fruits remained healthy during ripening. The DS treatment was almost as effective after 4 weeks storage, but after 6 weeks of storage woolliness began to develop. In contrast, the amount of healthy fruit in the control treatment after 3 days ripening was 50% (4 weeks) and 0% (6 weeks). The subsequent increase in the percentage of healthy fruits during ripening has been previously reported for 'Independence' nectarines (Von Mol-

lendorff et al., 1989, 1992). Von Mollendorff et al. (1992) suggested that limited degradation of the pectin during ripening may lead to a temporary state of woolliness, which further cell wall hydrolysis dissipated.

The changes in expressible juice also corresponded to the percentage of healthy fruits in which higher expressible juice was associated with a greater number of healthy fruits (Fig. 1(c,d)). At removal from 4 and 6 weeks storage, DS fruit were less firm than control and CA storage fruits (Table 1, Fig. 1(e,f)). DS and CA fruit had similar SSC but higher TA than control fruits at both removals (Table 1).

Peaches stored in CA generally retain better quality than air-stored fruits and many effective gas concentrations have been reported (Tonini et al., 1989). Concentrations of CO<sub>2</sub> ranging from 5 to 20%, combined with low O<sub>2</sub>, have been beneficial in preventing chilling injury in nectarines and peaches (Wade, 1981; Tonini et al., 1989; Lurie, 1992). High CO<sub>2</sub> delayed fruit ripening, keeping the fruit firmer, and preventing the development of woolliness in nectarines (Lurie, 1992; Retamales et al., 1992). In this study, CA storage with 10% CO<sub>2</sub> and 3% O<sub>2</sub> was most effective in preventing chilling injury and maintaining quality amongst the three treatments.

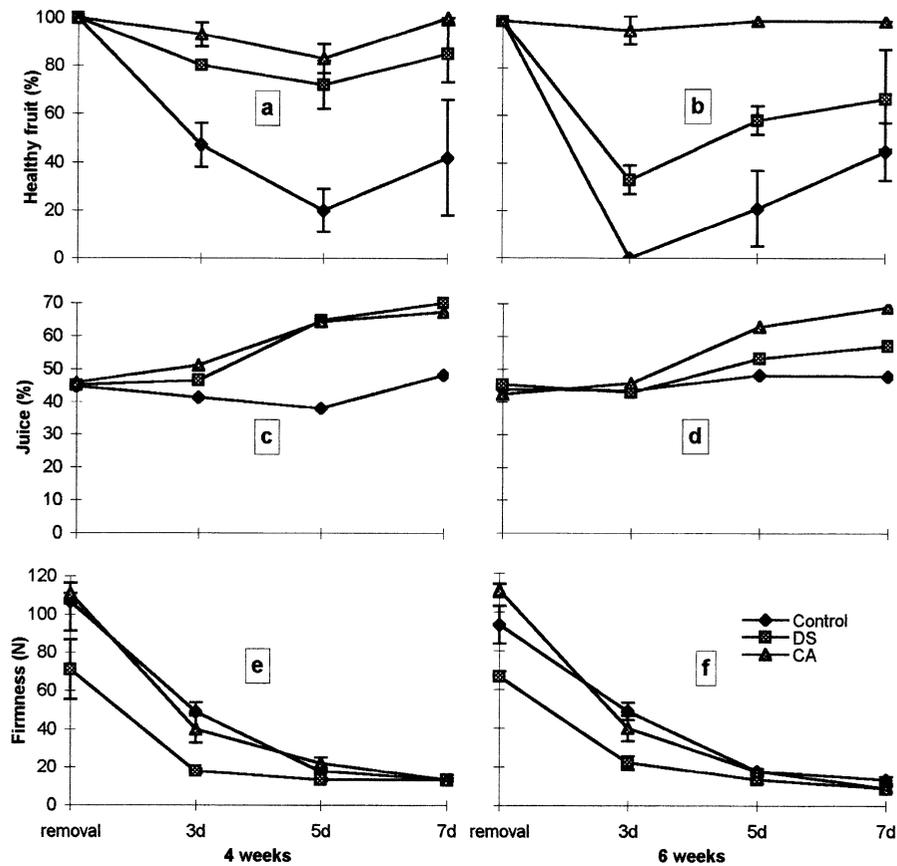


Fig. 1. 'Flavortop' nectarine fruit stored for 4 or 6 weeks and then ripened for 7 days at 20°C shelf life. Fruit with healthy flesh after 4 weeks (a) and 6 weeks (b); expressible juice from fruits after 4 weeks (c) and 6 weeks (d); firmness of fruits after 4 weeks (e) and 6 weeks (f).

Table 1  
Ripeness parameters of nectarines following 4 or 6 weeks of storage

	Firmness (Newton)	SSC (%)	TA (%)
<i>4 weeks:</i>			
Control	107.9 ± 4.3	11.0 ± 0.2	0.70 ± 0.016
Delayed stor- age	70.9 ± 15.7	11.7 ± 0.1	0.89 ± 0.026
Controlled at- mosphere	109.1 ± 5.2	10.5 ± 0.1	0.81 ± 0.012
<i>6 weeks:</i>			
Control	93.7 ± 9.7	11.0 ± 0.4	0.67 ± 0.034
Delayed stor- age	68.3 ± 2.5	11.4 ± 0.3	0.90 ± 0.037
Controlled at- mosphere	110.5 ± 3.8	11.3 ± 0.5	0.89 ± 0.025

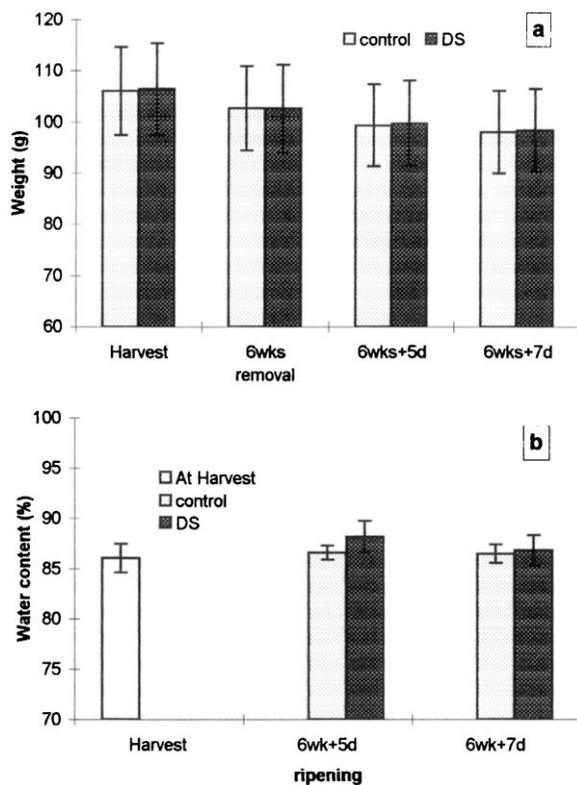


Fig. 2. 'Flavortop' nectarine fruit at harvest and following storage for 6 weeks. Fruit weight (a) and fruit water content (b).

### 3.2. DS and weight loss

DS fruits had the same weight loss at the end of 6 weeks storage as control fruits (Fig. 2(a)). In both cases weight loss continued at a similar rate during post storage ripening.

The total water content of DS fruit during ripening following storage was no different from that in control fruit (Fig. 2(b)). Ben-Arie and Lavee (1971) showed that the loss of juiciness is unconnected with the loss of water from woolly peaches and that the amount of expressible juice from woolly fruit increases when the fruit is heated. Our result in which neither fruit weight nor total water content was significantly different between control and DS confirmed that less juice in woolly fruits was not due to loss of water from the fruits; rather that the water was in some way bound. It has been suggested to be bound in the pectin fraction of the cell walls (Ben-Arie and Lavee, 1971).

### 3.3. Softening with PG and EGase

Some studies have shown that DS causes more softening and weight loss than control fruits (Tonini et al., 1989; Retamales et al., 1992). We also found that fruit from DS at removal after 4 and 6 weeks storage were softer than fruit from control and CA storage (Table 1). However, during 5 days ripening, fruit firmness from the three treatments decreased to a similar level, beyond which the fruit did not soften any further (Fig. 1(e,f)).

Rapid softening is associated with PG activity which degrades the pectin in the cell wall matrix, causing a decrease in cell wall coherence leading to softening (Pressey et al., 1971; Crookes and Grierson, 1983; Huber, 1983). Both exo- and endo-PG activities were higher in DS fruit than in control fruit upon removal from storage (Table 2). This might be related to the lower firmness of these fruit at removal. The rapid increase in exo-PG activity in both DS and CA treatments, during ripening after 4 weeks storage far exceeded the increased activity in control fruit. After 6 weeks storage when woolliness developed in DS fruit (Fig. 1), exo-PG activity declined to a similar level

as that in control fruit after 4 weeks, but in CA fruit PG continued to be very active. These differences were not related to the firmness of the fruits from all treatments, which decreased to a similar level of softness. Therefore, softening might be related more to endo-PG, which is more efficient in cleaving an interior site in the pectin molecule, leading to a rapid decrease in molecular mass (Pressey et al., 1971). Its activity in the softer DS fruit was higher upon removal from storage than in fruit of the other treatments, and during ripening, endo-PG activity increased to similar levels in all three treatments. On the other hand, EGase in control fruit did not change during ripening after both removals and was therefore higher than in fruits from DS and CA storage where its activity was reduced. Metabolism by EGase of xyloglucan closely associated with cellulose microfibrils has been considered to contribute to the initial stages of fruit softening (Rose et al., 1998). In our study, the EGase appeared to be related more to the development of woolliness than to softening and the general trend was for a decrease in its activity as the fruit ripened.

#### 3.4. *Woolliness and the balance between PG and PE enzymes*

Woolliness in peaches is postulated to be due to imbalance of PG and PE activities (Ben-Arie and Sonogo, 1980). When this occurs, polygalacturonic acid, the major component of pectin, is de-esterified without subsequent degradation, resulting in large pectin molecules with reduced methylation. Polygalacturonan with fewer esterification groups can form a low ester gel (Thakur et al., 1997). In this study control fruit had the lowest PG/PE ratio in shelf life (Table 2). This was consistent with results from peaches (Ben-Arie and Sonogo, 1980; Artes et al., 1996). Under this imbalance of PG and PE, it is likely that gelling could cause more free water to be bound into gel, leading to less 'free juice' and causing woolliness. However, there was no difference in PG and PE mRNA expression between control and delayed cooling storage (Fig. 3). This implied that the relatively lower PG and higher PE activities in control fruit were not due to blocking of

mRNA transcription. The differences might therefore either be at the step of translation or inhibition of enzyme activities under chilling temperature and subsequent ripening.

CA treatments provided a high percentage of healthy fruits (Fig. 1). However, activities of exo-PG and endo-PG were as low during storage as in control fruit (Table 2). On the other hand, PE activity was also reduced during CA storage. After 5 days ripening, exo-PG activity in CA fruit was much higher than in fruit from the two air storage treatments and endo-PG showed similar activity in all treatments. With relatively lower PE activities, CA resulted in the highest PG/PE ratio among the three treatments during ripening (Table 2). This indicated that a rapid rise in PG activity and a high PG/PE activity during ripening may play a critical role in preventing woolliness. This could be the reason that after CA storage most of the fruits were free of woolliness. Upon removal from CA to 20°C air, the recovery of PG activity enabled pectin molecules to be cleaved quickly during ripening. With lower PE activity, the extent of demethylated pectic substances was suited to the increased level of PG activity, so that the substrate for gel formation was not produced and woolliness did not occur.

High CO<sub>2</sub> and low O<sub>2</sub> can protect peach and nectarine fruits from chilling injury (Wade, 1981; Anderson, 1982; Tonini et al., 1989; Lurie, 1992; Retamales et al., 1992). Low PG at removal could be due to the effect of high CO<sub>2</sub> and low O<sub>2</sub> on enzyme synthesis. It is reported that high CO<sub>2</sub> reduced the synthesis of enzymes such as ATP:phosphofructokinase (PFK), PPi:phosphofructokinase (PFP), and ACC synthase (Kerbel et al., 1988; Gorny and Kader, 1993). Our results indicate that CA with high CO<sub>2</sub> and low O<sub>2</sub> inhibited PG at the transcription level (Fig. 3). At removal from cold storage there was almost no PG mRNA detected in CA-stored fruit, although it increased rapidly during ripening along with its increase in activity. Compared to regular air storage the mRNA of endo-PG of CA-stored fruits at removal was much repressed. In contrast, the mRNA level of PE was similar in fruits from all three treatments; it was low at removal and increased during ripening. This is the

opposite of PE activity, which decreased during ripening.

Bramlage (1982) suggested that the benefit from DS is due to the more advanced stage of ripeness when cold storage is begun, since chilling interferes with the ability of the fruit to ripen. CA with high CO<sub>2</sub> can conserve the ripening ability at chilling temperatures and subsequently enable normal ripening (Lurie, 1992). In our study, DS alleviation of chilling injury was associated with retention of greater PG activity during cold storage (as seen at removal), and normal increase in activity during ripening and higher PG/PE. It may

be that the 2 days at 20°C before storage switched on the ripening process and this process continued slowly at 0°C, thereby avoiding chilling injury. The high PG/PE is the result of this switched on ripening process and guarantees normal ripening. Therefore, DS and CA appear to prevent woolliness by different mechanisms; DS maintains enzyme activity during cold storage, while CA represses PG activity (and mRNA) but retains the ability to recover from the repression when the fruit are rewarmed.

Elevated EGase mRNA appears to be a result of chilling injury. There was almost no EGase

Table 2

Activities of exo-PG, endo-PG, PE, and endo-glucanase and the ratios between exo-PG to PE (endo-PG/PE) and endo-PG to PE (endo-PG/PE) after 4 or 6 weeks of storage and 5 days at 20°C

	4 weeks		6 weeks	
	Removal	+ 5d ripening	Removal	+ 5d ripening
<i>Exo-PG</i> <sup>a</sup>				
Control	83 ± 5*	273 ± 26	123 ± 8	203 ± 16
DS	116 ± 16	400 ± 24	138 ± 8	222 ± 18
CA	31 ± 4	477 ± 14	73 ± 10	460 ± 13
<i>Endo-PG</i> <sup>b</sup>				
Control	5 ± 0.6	19 ± 0.5	9 ± 1.3	13 ± 3.0
DS	13 ± 1.5	20 ± 0.3	12 ± 1.5	12.5 ± 1.7
CA	6 ± 0.6	18 ± 2.0	7 ± 0.2	14 ± 0.9
<i>PE</i> <sup>c</sup>				
Control	0.97 ± 0.021	0.65 ± 0.075	1.03 ± 0.088	0.66 ± 0.051
DS	0.85 ± 0.093	0.65 ± 0.030	0.90 ± 0.078	0.62 ± 0.070
CA	0.80 ± 0.026	0.56 ± 0.048	0.87 ± 0.105	0.53 ± 0.034
<i>Exo-PG/PE</i>				
Control	86	420	119	308
DS	136	615	153	358
CA	39	852	84	868
<i>Endo-PG/PE</i>				
Control	5.2	29.2	8.7	19.7
DS	15.3	30.8	13.3	20.2
CA	7.5	32.1	8.0	26.4
<i>EGase</i> <sup>d</sup>				
Control	2.0 ± 0.30	1.4 ± 0.12	1.3 ± 0.04	1.3 ± 0.02
DS	1.8 ± 0.30	1.0 ± 0.05	1.2 ± 0.02	1.0 ± 0.07
CA	1.9 ± 0.03	0.8 ± 0.07	1.4 ± 0.06	0.7 ± 0.13

<sup>a</sup> One unit exo-PG defined as 1 µg galacturonic acid released per mg protein/h.

<sup>b</sup> One unit of endo-PG defined as 1 s change in viscosity per mg protein/h.

<sup>c</sup> One unit of PE defined as 1 mM NaOH consumed per mg protein/h.

<sup>d</sup> One unit of EGase defined as 1 s change in viscosity per mg protein/h.

\* Standard deviation.

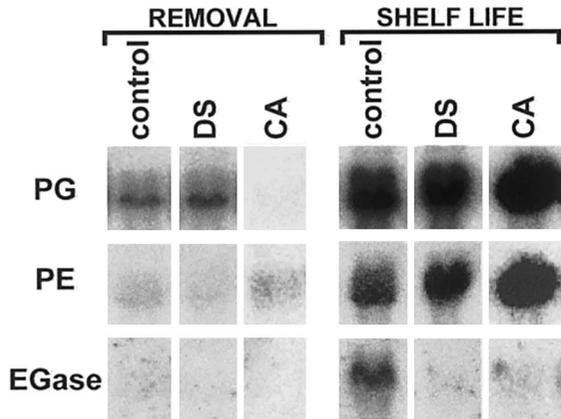


Fig. 3. Changes in mRNA levels of polygalacturonase (PG), pectin esterase (PE) and  $\beta$ -1,4-endoglucanase (EGase) at removal from 4 weeks storage and after ripening for 5 days at 20°C. Fruit were held in regular air 0°C storage (control), held for 48 h at 20°C before 0°C storage (DS), or stored in 3% O<sub>2</sub>, 10% CO<sub>2</sub> controlled atmosphere storage (CA).

mRNA expression at all times of observation in all treatments, except with the development of woolliness, when EGase mRNA was elevated. Elevated mRNA for EGase was also found after 6 weeks in regular air control and DS fruits but not in fruit from CA (data not shown). During normal ripening it is necessary to amplify total RNA by RT-PCR in order to observe the EGase message (Bonghi et al., 1998). The large increase in message in woolly fruits is of interest, but currently there is no explanation for it. It does not appear to lead to an increase in EGase activity, which tends to decrease as the fruit ripens.

#### 4. Conclusion

DS alleviated and CA storage prevented woolliness (chilling injury) in nectarine fruits. The results were related to the relative activities of PG and PE. DS maintained a level of PG activity during storage, which allowed for continuous increase following storage and normal softening. CA storage, however, repressed PG mRNA and enzyme activity during storage, but PG activity increased quickly during ripening. Both DS and CA storage led to a high PG/PE ratio of activity

in fruits during ripening which coordinated both demethylation and cleavage of pectin, leading to normal ripening. An increase in EGase mRNA expression seemed to be linked to chilling injury.

#### Acknowledgements

This research was partially funded by BARD: Binational Agricultural Research and Development Fund between the United States and Israel.

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