VOL. 5, NO. 5, SEPTEMBER 2010 ISSN 1990-6145
ARPN Journal of Agricultural and Biological Science


www.arpnjournals.com

REGULATION OF CELLULASE AND PECTINASE ACTIVITIES IN CHERRY TOMATO (*Lycopersicon esculentum* MILL var. *cerasiforme*) FRUIT BY USE OF 1-METHYLCYCLOPROPENE

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ABSTRACT

Softening, which accompanies ripening fruits is also to a large extent, responsible for the damage that occurs during handling and shipping. The effects of 1-Methylcyclopropene (1-MCP) application at different ripening stages (mature green, breaker, breaker + 2 days and breaker + 4 days) on cellulase and pectinase activities of cherry tomato fruit (*Lycopersicon esculentum* Mill. *var. cerasiforme*) were studied. Fruit were exposed to a $5\mu l^{-1}$ 1-MCP concentration and enzyme activity was determined viscometrically. Both cellulase and pectinase activities were suppressed by 1-MCP treatment, especially in MG and BR fruit. 1-MCP delayed cellulase activity peak by at least 3 days and 6 days in treated MG and BR fruit, respectively compared to their corresponding controls. In treated MG fruit, pectinase activity was suppressed and the initiation delayed by 3 days compared to untreated fruit. Results indicate that 1-MCP when applied at either MG or BR stages of ripening is beneficial for at least 10 days.

Keywords: cherry tomato fruit, cellulose, pectinase activities, ethylene antagonist, fruit softening enzymes, quality.

1. INTRODUCTION

Fruit softening during ripening is a major cause of post harvest losses due to damage incurred during handling and shipping. As a result of this, post harvest biologists have studied fruit softening with the aim of identifying ways of how best to manage ripening without compromising fruit quality. During fruit ripening, the primary cell wall undergoes structural changes as the fruit softens. Softening, according to Chung *et al*., (2006), is thought to be associated with the degradation of pectic substances in the cell caused by accumulation of cell wall hydrolase activities such as pectinesterase (PE), polygalacturonase (PG) and β-galactosidase. It is believed that fruit softening during ripening is controlled by a cellulase that degrades the cell wall cellulose and polygalacturonase(s) that degrade middle lamella pectin (Abeles and Takeda, 1990). The two cell wall degrading enzymes most frequently associated with fruit softening are cellulase and polygalacturonase (PG).

Cellulase is considered to be one of the most important enzymes involved in the degradation of cell wall polysaccharides. However, according to Abu-Goukh and Bashir (2003), the role of cellulase in fruit softening is uncertain. Huber (1983) also reported that cellulase are ubiquitously distributed in ripening fruit, and are associated with softening, but their precise role in fruit softening is not clear. Softening during ripening in climacteric fruit is generally attributed to degradation in cell wall assembly, particularly the solublization of pectin; and the changes could involve increased activities of various cell wall hydrolases. According to Majumder and Mazumdar (2002), pectic substances are the major components of the cell wall and middle lamella of plant tissues which undergo structural changes during development and ripening of the fruit, thus contributing significantly to textural softening of these organs. Due to the hypothesized structural role of pectin in plant tissue rigidity, the loss of pectin integrity during tomato fruit ripening has been implicated as the predominant component of ripening associated softening. Softening of most fruits is accompanied by a decrease in total pectin and by an increase in water-soluble pectin. Ripening fruit softens because pectin and other cell wall carbohydrates are broken down enzymatically. Pectin is degraded by a group of pectinases; which are a group of enzymes and includes polygalacturonases, pectinmethylesterases and pectin lyases. Pectinases are produced during the natural ripening process of some fruit.

Ethylene is necessary for the ripening process in climacteric fruit, and has been implicated in fruit softening. In avocado and tomato, ethylene has been reported to control cellulase synthesis (Abeles and Takeda, 1990). Various factors including the fruit ripening stage influence the cell wall degrading enzymes activities. Abeles and Takeda (1990), found that in strawberry, cellulase activity increased during growth and ripening; with the largest increase occurring in overripe fruit; but there was no increase in polygalacturonase and pectinase activity. Cellulase activity was high in unripe durian aril, but remained constant throughout softening (Ketsa and Daengkanit, 1999). In durian fruit, ripening is accompanied by increased activity of polygalacturonase and β-galactosidase; but pectinesterase does not seem to be rate limiting for softening since its activity is usually already high at harvest (Imsabai *et al.,* 2002).

Polygalacturonase has been considered a primary enzyme regulating fruit softening, but its role in tomato softening is questionable since transgenic tomato fruit having only 1% of the original fruit specific polygalacturonase activity levels softened normally (Smith

et al., 1988). In an experiment on bananas, Lohani *et al.,* (2004) reported that ethylene stimulated the activities of pectinmethylesterase (PME), polygalacturonase (PG), pectin lyase (PL) and cellulase; while 1-MCP treated fruit did not exhibit marked changes in firmness or increases in activities of these cell wall hydrolases.

Although the mechanisms by which fruits soften during ripening remain unclear, enzyme-catalyzed changes to wall structure and composition are believed to be the major causes. Taking into consideration the economic consequences of post harvest fruit softening, the purpose of this study was to determine whether the delay/suppression of loss in fruit softening due to 1-MCP application as reported by Opiyo and Ying (2005) was as a result of inhibition of cell wall enzymes activities. It was of interest to determine how 1-MCP might regulate cellulase and pectinase activities, and at what ripening stage this occurs in cherry tomato.

2. MATERIALS AND METHODS

2.1. Experimental material

The experiment was conducted at the Food Science Department of Zhejiang University with cherry tomato fruit obtained from the Zhejiang Academy of Agricultural Sciences. Fruit were harvested at either mature green (MG) or breaker (BR) stage of ripening and left overnight at room temperature in order to check any rise in ethylene evolution due to wounding. Thereafter the fruit were sorted out based on size and freedom from blemishes. The average fruit weight was 10 ± 2g. 1-MCP was generated from Smart Fresh™ (0.14 a. i.) by putting a predetermined weight in a glass bottle and adding water. The bottle was immediately sealed and allowed to stand for 1 h. Fruit were subjected to a 1-MCP concentration of $5\mu l^{-1}$ in this experiment. Fifty fruit at different ripening stages were placed in 4L desiccators and sealed. The desired 1-MCP concentration was injected into the desiccators, except the control into which an equal volume of air was injected. There were eight treatments namely;

a) Mature Green (MG)
b) Mature Green + 1-MCP (MG + 1-MCP)
c) Breaker (BR)
d) Breaker + 1-MCP (BR + 1-MCP)
e) Breaker + 2 days (BR + 2)
f) Breaker + 2 + 1-MCP (BR + 2 + 1-MCP)
g) Breaker + 4 (BR + 2)
h) Breaker + 4 + 1-MCP (BR + 4 + 1-MCP)

To obtain fruit at BR + 2 and BR + 4 ripening stages, fruit were harvested at breaker stage and held for 2 and 4 days respectively before being treated with 1-MCP. Fruits were exposed to 1-MCP for a period of 22 h; after which fruit (15 fruits per treatment per replicate) were placed in perforated plastic trays and stored in an incubator at a temperature of 22°C and a RH of 85% for a period of two weeks.

2.2. Experimental design

The experiment consisted of 8 treatments arranged in a "Randomized Complete Block Design" (RCBD); and was replicated thrice. Statistical design for ANOVA was done using the SAS GLM v. 6.12 computer program; and means separated by the "Tukey's Test". The level of significance was set at $P \leq 0.05$.

2.3. Determination of cellulase and pectinase activities

Cellulase activity was determined according to the method by Durbin and Lewis, (1988) with some modifications. The crude protein extraction buffer consisted of 10mM Na_2HPO_4/NaH_2PO_4, 10mM mercaptoethanol, 1mM Na_2-EDTA, 0.1% triton X-100 and 0.15M NaCl. 1g of frozen fruit pericarp was homogenized with 2ml of the extraction buffer in an ice cooled mortar. The mixture was then centrifuged at 10000g for 10 minutes at 4°C. After centrifugation, 0.8ml of 0.3% sodium carboxymethyl cellulose (CMC) was added to 0.4ml of the enzyme extract (supernatant after centrifugation) and mixed well. The mixture was then incubated at 30°C for 1 hour. For the control, the enzyme extract was substituted with 0.4ml of distilled water. To determine viscosity, the time needed for 200µl of the reaction mixture to pass through a micro-viscometer (0.01 cm^3/N 20°C B.S. 1428 U.K.) was determined using a stopwatch. Viscosity data were converted to intrinsic viscosity and relative units of activity; providing a linear relationship between viscosity and enzyme activity. The linearized cellulase activity unit was calculated as follows (Durbin and Lewis, 1988);
Linearized cellulase activity Unit:

$$B = \{(\eta^{-\alpha}-\eta_0^{-\alpha})\ Cs\}/t$$

Where:

B --- Linearized cellulase activity Unit
η --- Intrinsic viscosity after incubation, $= 8\ (T/T_0 - 1)^{1/8}/Cs$
η_0 --- Intrinsic viscosity before incubation, $= 8\ (T/T_0 - 1)^{1/8}/Cs$
Cs --- Substrate concentration, g/l;
t --- Incubation time, s;
T --- Time needed for reaction mix to pass through viscometer, s;
T_0 --- Time needed for control mix to pass through viscometer, s;
A --- Substrate constant, for CMC $_7$HP, α=3.66

The pectinase activity was determined in a similar fashion. However, the viscometric assay was performed with 1% pectin solution (pectin was of SIGMA from Sigma chemical co. U.S.A and contained 79.5% galacturonic acid and 8.1% methoxy). Data was expressed as relative activity, defined as percent reduction in viscosity of pectin solutions by pectinase extract compared with controls without enzyme extracts (Abeles and Takeda, 1990).

All assays were run in duplicate and the results presented are the average of two assays.

3. RESULTS

3.1. Cellulase activity

In MG and BR fruit the cellulase activity peak appeared at the 10th d of storage; and 1-MCP significantly suppressed the activity and delayed the peak. The effect of 1-MCP at later fruit ripening stages (BR + 2 and BR + 4) was not significant. The interaction between 1-MCP and storage duration significantly influenced cellulase activity, just as the interaction between 1-MCP, maturity stage and time significantly affected the cellulase activity in cherry tomato fruit. The interaction between fruit maturity stage and 1-MCP treatment was also not found to be significant. The individual effects of maturity stage, storage duration as well as their interaction on cellulase activity were all highly significant (Table-1).

Table-1. Effect of 1-MCP and fruit ripening stage on the cellulase activity of cherry tomato fruit.

	Days after treatment					
Maturity stage	**1**	**4**	**7**	**10**	**13**	**16**
MG	$2.92^{a,1}$	$5.48^{a,2}$	$6.42^{a,5}$	$185.17^{i,6}$	$16.05^{a,7}$	$29.76^{a,8}$
MG + 1-MCP	$3.28^{b,1}$	$4.62^{b,2}$	$2.13^{b,5}$	$35.00^{b,9}$	$91.47^{j,11}$	$46.31^{b,8}$
BR	$3.25^{c,1}$	$6.84^{c,2}$	$4.12^{c,5}$	$106.07^{k,10}$	$17.00^{c,7}$	$22.58^{c,8}$
BR + 1-MCP	$7.67^{d,1}$	$5.68^{d,2}$	$2.13^{d,5}$	$69.10^{d,9}$	$51.76^{d,7}$	$89.86^{m,8}$
BR + 2	$6.75^{e,1}$	$74.12^{n,3}$	$27.14^{e,5}$	$28.46^{e,9}$	$18.38^{e,7}$	$10.58^{e,8}$
BR + 2 + 1-MCP	$5.67^{f,1}$	$213.08^{p,4}$	$52.14^{f,5}$	$40.92^{f,9}$	$6.93^{f,7}$	$51.89^{f,8}$
BR + 4	$21.56^{g,1}$	$7.91^{g,2}$	$11.37^{g,5}$	$6.08^{g,9}$	$9.45^{g,7}$	$10.35^{g,8}$
BR + 4 + 1-MCP	$34.89^{h,1}$	$7.02^{h,2}$	$13.52^{h,5}$	$11.75^{h,9}$	$13.45^{h,7}$	$8.32^{h,8}$

Values with the same letter along a row and those with the same number down a column are not significantly different at P≤0.05 according to Tukey's test.

The peak of cellulase activity in 1-MCP treated MG fruit occurred 13 days and in untreated fruit 10 days after treatment (Figure-1).

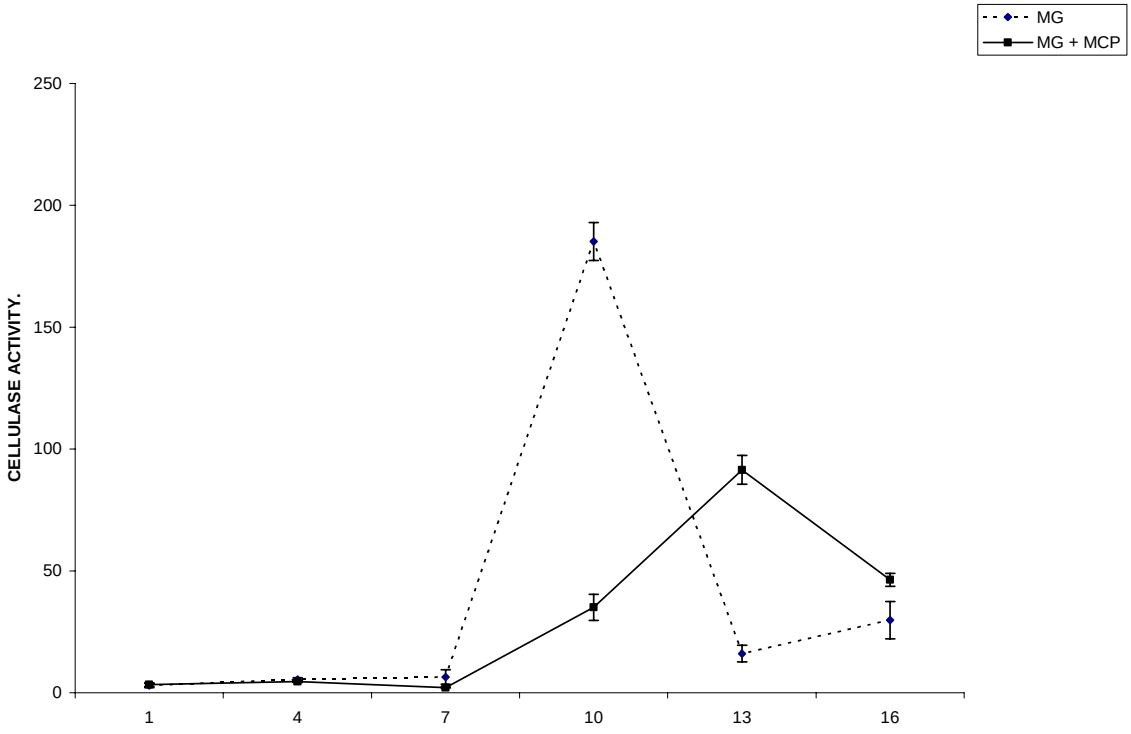

Figure-1. Effect of 1-MCP on cellulase activity of MG cherry tomato fruits. Error bars represent the SE of the mean of three replications.

In 1-MCP treated BR fruit, peak occurred 16 days after treatment, 6days later then the control and the peak was also lower (Figure-2).

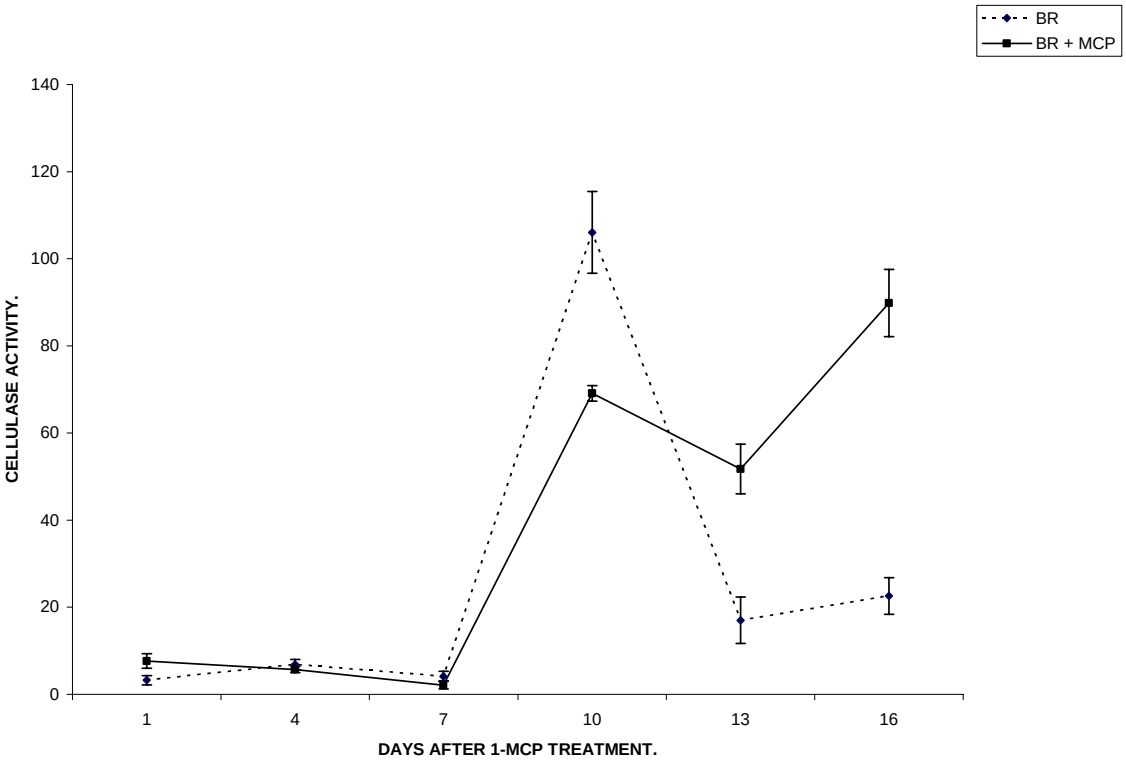

Figure-2. Effect of 1-MCP on cellulase activity of BR cherry tomato fruits. Error bars represent the SE of the mean of three replications.

Both treated and untreated BR + 2 fruits exhibited the cellulase activity peaks 4 days after treatment; (Figure-3).

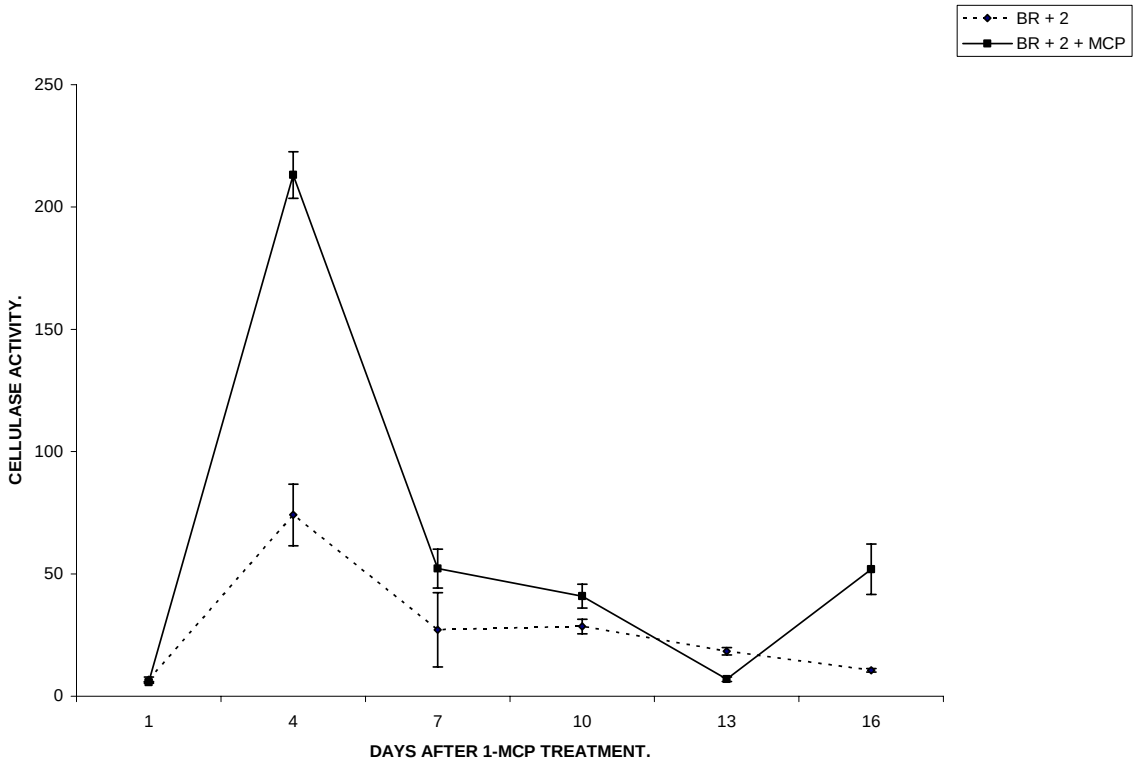

Figure-3. Effect of 1-MCP on cellulase activity of BR + 2 cherry tomato fruits. Error bars represent the SE of the mean of three replications.

Cellulase activity in BR + 4 fruit was already high at the start of storage, and declined right from 1 day after treatment, (Figure-4). 1-MCP appeared to have no effect on cellulase activity at these later ripening stages.

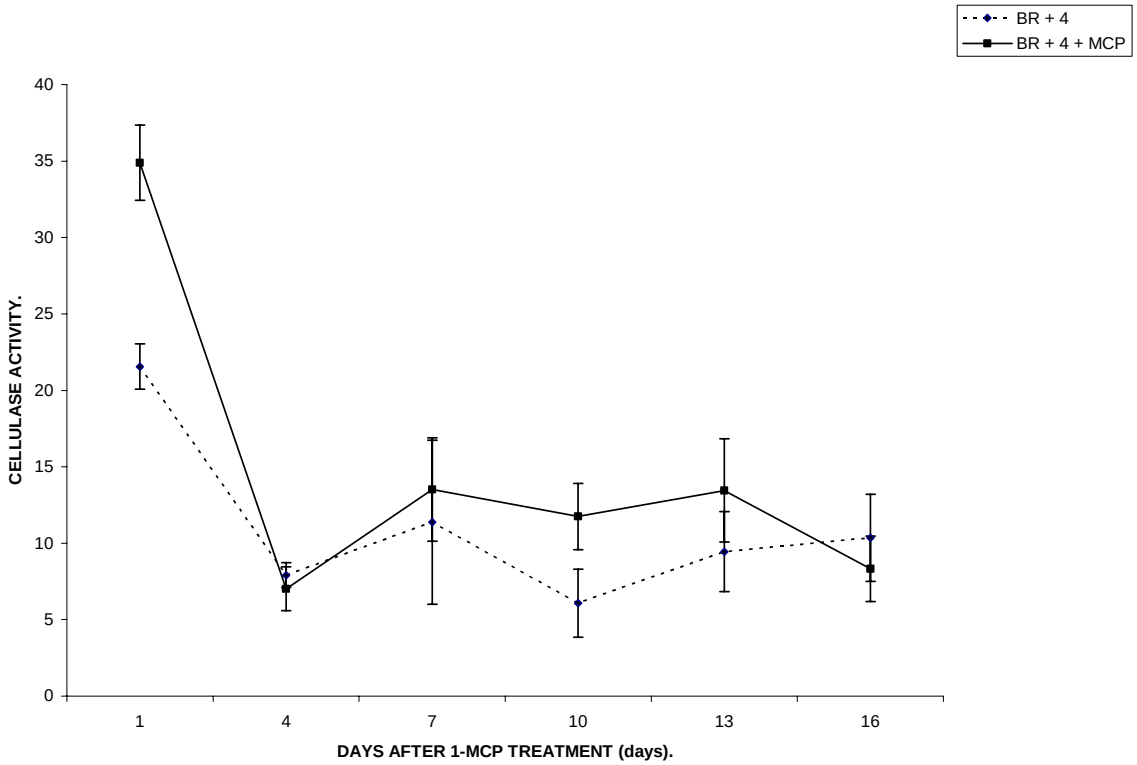

Figure-4. Effect of 1-MCP on cellulase activity of BR + 4 cherry tomato fruits. Error bars represent the SE of the mean of three replications.

3.2. Pectinase activity

1-MCP treatment and fruit ripening stage had significantly high effects on pectinase activity. However, the interaction of 1-MCP and maturity, 1-MCP and time, 1-MCP and maturity and time all had no significant effects on pectinase activity. In MG fruit, there was little difference in activity between 1-MCP treated and untreated fruit during the first 7 days after treatment. In untreated MG fruit, pectinase activity was initiated 7 days after treatment; while in 1-MCP treated fruit initiation was 10 days after treatment; i.e. 3 days later than in untreated fruits (Figure-5). 10 days after treatment, activity in untreated fruit was significantly higher than in treated fruit. The pectinase activity was also suppressed in treated fruit compared to untreated fruit.

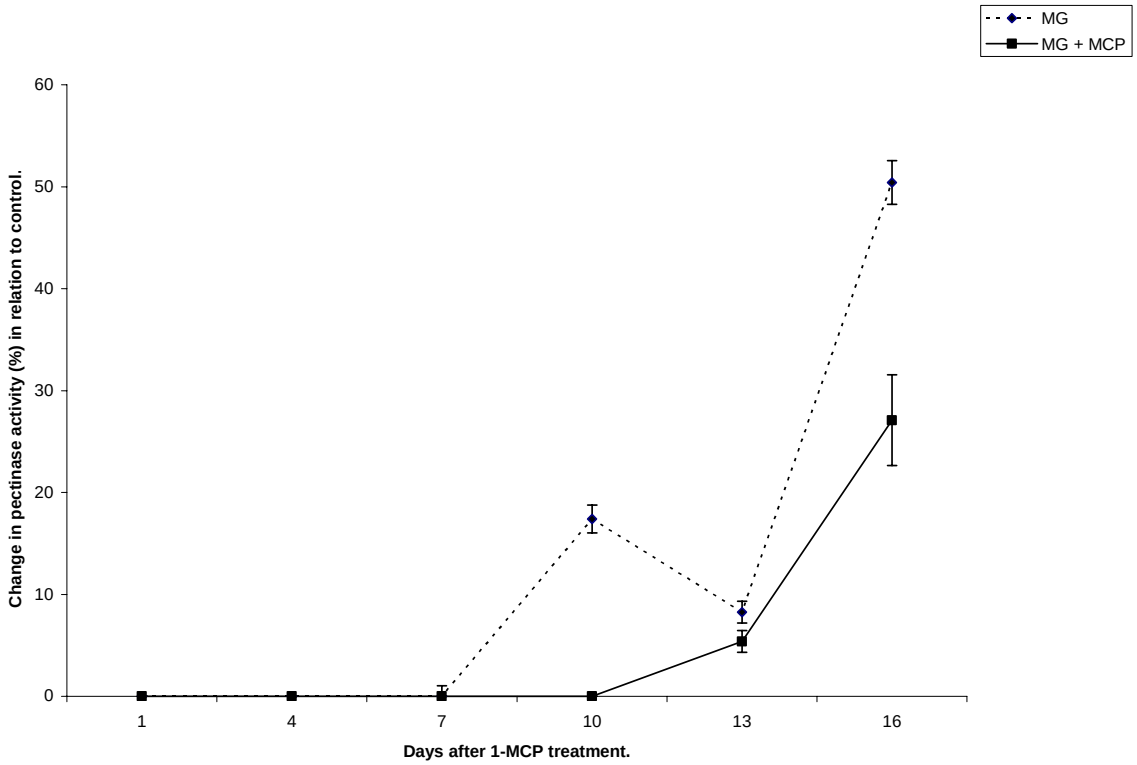

Figure-5. Effect of 1-MCP on pectinase activity of MG cherry tomato fruit. Error bars represent the SE of the mean of three replications.

The trend in BR fruit was not very different from that in MG fruit. Just as in MG fruit, 1-MCP treated BR fruit exhibited a lower activity compared to untreated fruit, though in most cases the differences were not significant. Pectinase activity was initiated 7 days after treatment in both treated and untreated fruit (Figure-6). Although 1-MCP did not delay the pectinase initiation in BR fruit, it did suppress its activity. 10 days after treatment, pectinase activity in 1-MCP treated fruits was significantly higher than in untreated fruit.

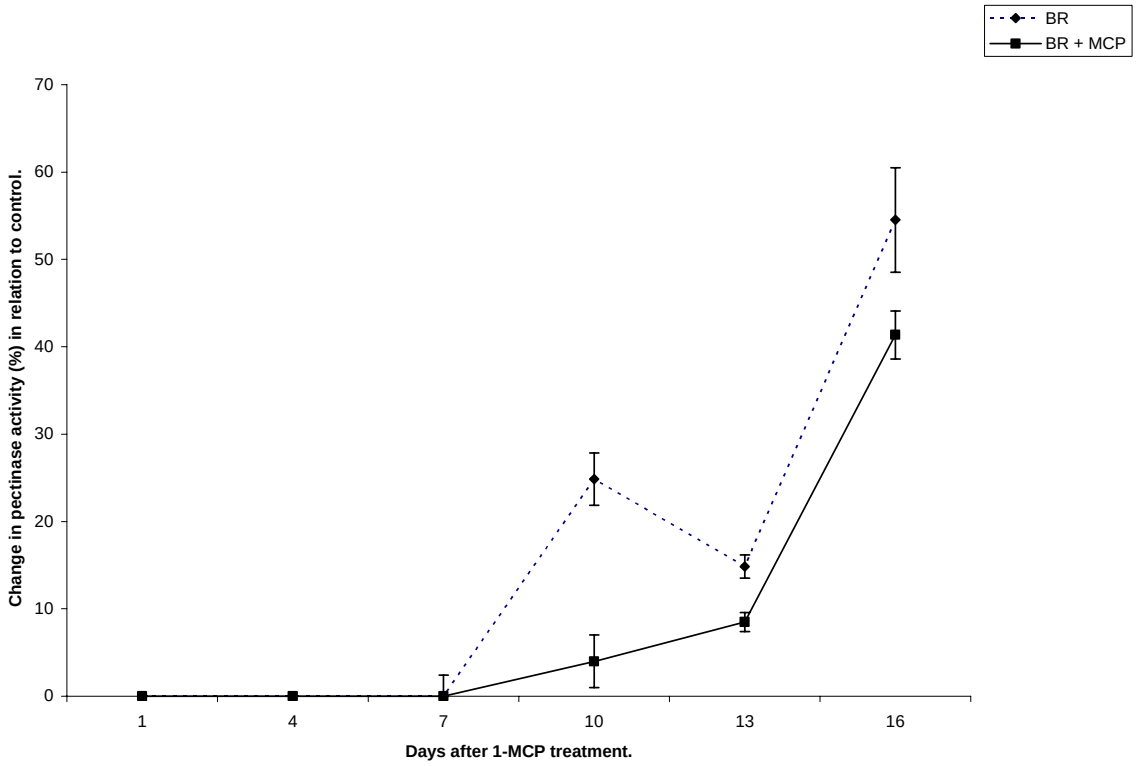

Figure-6. Effect of 1-MCP on pectinase activity of BR cherry tomato fruit. Error bars represent the SE of the mean of three replications.

By the start of the storage period, pectinase activity in both treated and untreated BR + 2 fruit was already high. Pectinase activity in BR + 2 fruits (treated and untreated) was significantly higher compared to MG and BR fruit 1 day after treatment. 1-MCP treated BR + 2 fruit exhibited lower activity compared to untreated fruit (Figure-7). After an initial increase in activity during the first 4 days, there was a sharp decline in activity (especially in treated fruit) between 4 and 10 days after treatment. This was followed by a sharp increase in activity in both treated and untreated fruit. Throughout the period of storage differences in activity between treated and untreated BR + 2 fruit were not significant.

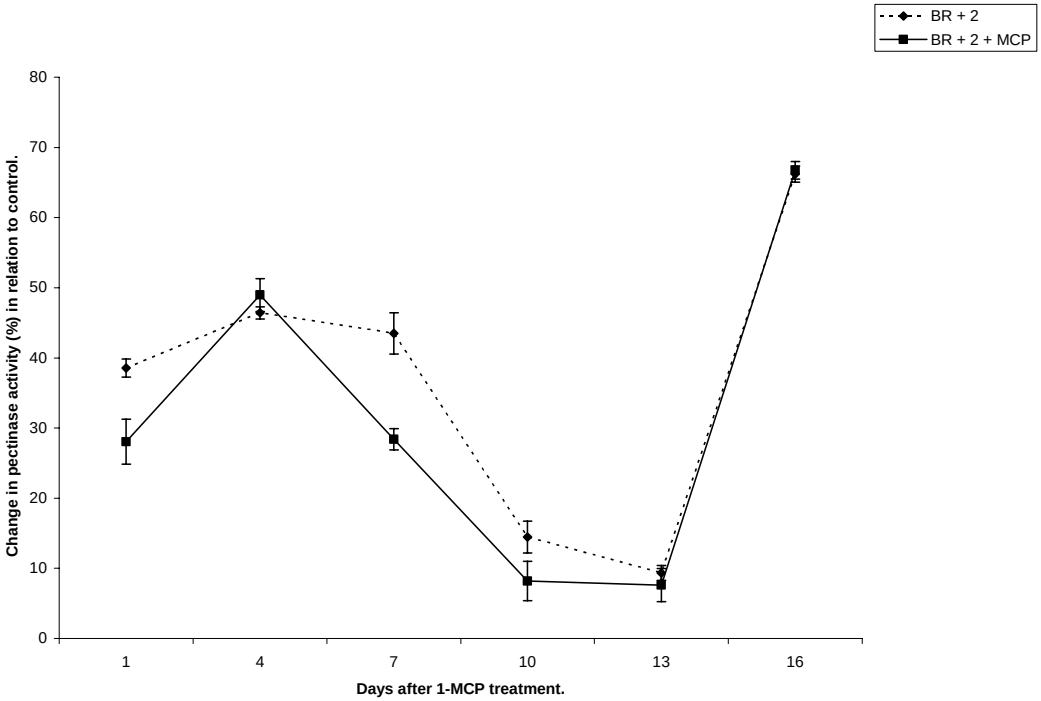

Figure-7. Effect of 1-MCP on pectinase activity of BR + 2 cherry tomato fruit. Error bars represent the SE of the mean of three replications.

The activity trend in BR + 4 fruit was more-or-less similar to that of BR + 2 fruit (Figure-8). Activity declined in both treated and untreated fruit during the first 10 days of storage; this was followed by a marked increase in activity between 10 and 16 days after treatment. Although treated fruit had a lower activity rate than untreated fruit, at no time during storage were the differences significant.

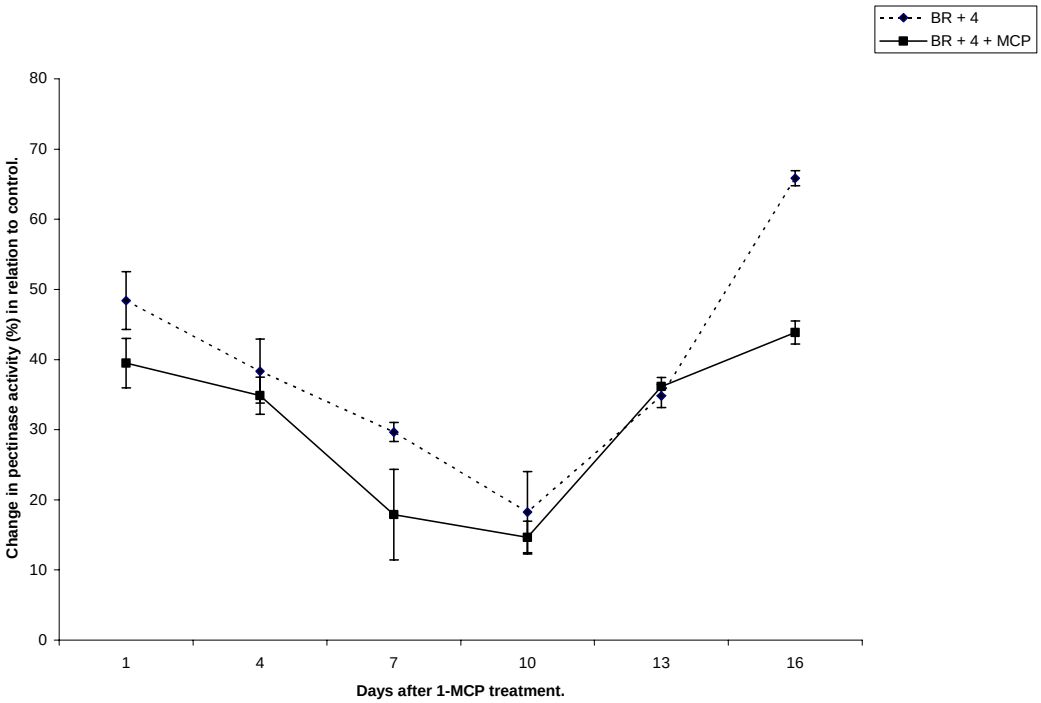

Figure-8. Effect of 1-MCP on pectinase activity of BR + 4 cherry tomato fruit. Error bars represent the SE of the mean of three replications.

4. DISCUSSIONS

The two cell wall - degrading enzymes most frequently associated with fruit softening are cellulase and polygalacturonase (PG). In the present study, cellulase and pectinase activities were examined to further analyze the effect of 1-MCP in delaying cherry tomato fruit ripening. Although cellulase activity initiation was not delayed by 1-MCP, we observed that activity was suppressed. Cellulase activity in untreated fruit increased ≈ 63 fold in MG fruit between 1 and 10 days (activity peak) after treatment. In 1-MCP treated MG fruit activity increased ≈ 11 fold during the same period; and ≈ 28 fold between 1 and 13 days (activity peak) after treatment. In BR fruit, activity in untreated fruit increased ≈ 33 fold between 1 and 10 days (activity peak) after treatment; while in treated fruit activity increased ≈ 9 fold during the same period, and ≈ 12 fold between 1 and 16 days (activity peak) after treatment. In untreated BR + 2 increase in activity was ≈ 11 fold and in treated fruit it was ≈ 38 fold between 1 and 4 days (activity peaks).

Our observation that cellulase activity increased with increasing maturity stage are in conformity to those reported by several authors in various species including avocado (Pesis *et al.,* 1978), capsicums (Sethu *et al.,* 1995) sweet cherry (Choi *et al.,* 2002), guava (Abu-Goukh and Bashir, 2003) and banana (Lohani *et al.,* 2004). According to Huber (1983), cellulase activity is usually low or undetectable in unripe fruit, but increases dramatically during ripening and softening. Low cellulase activity in 1-MCP treated fruit has previously been reported in avocado (Feng, *et al.,* 2000).

We observed that 1-MCP suppressed cellulase activity, and the activity curve in MG and BR fruit bore resemblance to the typical ethylene evolution curve. On the basis of these two observations, we can deduce the involvement of ethylene in cellulase activity. In post-breaker (BR + 2 and BR + 4) fruit, differences between 1-MCP treated and untreated fruit were not significant. It would appear that at the time of 1-MCP treatment application to BR + 2 fruit, cellulase activity was already in progress and approaching its peak. It is possible that the 1-MCP concentration was not enough to suppress the activity already in progress. In BR + 4 fruit, it appears that the cellulase activity was already past its peak and was on the decline. It is reasonable to suggest that application of 1-MCP after or soon before the cellulase activity peak has minimal effect in delaying cellulase effect on cell wall degradation.

Pectinases are a mixture of enzymes and includes polygalacturonase, pectin methylesterase and pectin lyases. Polygalacturonase is a fruit ripening specific cell wall pectinase; and in the ripening and softening process its activity has been strongly implicated as a key determinant of softening during tomato fruit ripening (Giovannoni *et al.,* 1992). Although the effect of 1-MCP on pectinase activity was highly significant, it did not completely inhibit activity, but rather just suppressed it. 1-MCP treatment was more effective in suppressing pectinase activity in MG and BR fruit than in BR + 2 and BR + 4 fruit. In BR + 2 and BR + 4 fruit, the pectinase activity was already high by the time of 1-MCP treatment; hence increase in activity was less compared to MG and BR fruit. Just as we observed that pectinase activity rate in BR + 2 and BR + 4 was lower compared to MG and BR fruit, Abeles and Biles (1991) reported that in apple fruit there was a reduction in activity or no activity when fruit were mature and ripe. Whereas in this study pectinase activity was suppressed but not completely inhibited by 1-MCP, Jeong and Huber (2003) observed that polygalacturonase (PG) activity was completely inhibited. In golden berry (*Physalis peruviana* L.) pectin methylesterase activity gradually increased as ripening proceeded (Trinchero *et al.,* 1999).

In this study the greatest increase in activity in the case of untreated MG and BR fruit took place between 4 and 10 days after treatment. This period coincides with the occurrence of the ethylene climacteric, and to some extent with that of maximum softening (as observed in an earlier study by Opiyo and Ying, (2005). In MG and BR fruit (treated and untreated), activity increased from 1 day after treatment and continued doing so until the experiment was terminated 16 days after treatment. This is in conformity with observations by Jeong *et al.,* (2002) that in avocado PG activity increased during the climacteric period and continued to increase during the post-climacteric phase; in addition 1-MCP delayed the decline in PME activity. Increase in PG activity has also been reported in *Capsicum annum* (Sethu *et al.,* 1995) and in guava (Abu-Goukh and Bashir, 2003). Increase in PME activity in tomato has previously been reported by Blumer *et al.,* (2000).

The observation that 1-MCP suppressed pectinase activity are consistent to a study by Jeong and Huber (2004) who reported that PG activity in 'Booth 7' avocado was strongly suppressed in 1-MCP treated fruit. Reduction in rate of PG activity in avocado due to 1-MCP treatment has also been reported by Feng *et al.,* (2000). In tomato Mostofi *et al.,* (2003) observed that PG activity was lower in 1-MCP treated fruit and suggested that it (1-MCP) directly inhibited ethylene-mediated changes in cell wall degrading enzyme activities.

5. CONCLUSIONS

The results of this study show that application of 1-MCP at a rate of $5\mu l.l^{-1}$ delayed the activities of both cellulase and pectinase. The effect of 1-MCP on cellulase appeared to be more pronounced compared to pectinase. We observed that the best results of 1-MCP on these cell wall enzymes were obtained with MG and BR fruit. In post-breaker fruit (BR + 2 and BR + 4), although 1-MCP suppressed pectinase activity, at no stage was the difference significant.

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