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BIOCHEMICAL CHANGES INVOLVED IN STRESS RESPONSE AND RIPENING BEHAVIOUR OF γ-IRRADIATED MANGO FRUIT

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Key Word Index—*Mangifera indica*; Anacardiaceae; Kent mango fruit; stress response; γ-radiation; ripening; phenylalanine ammonialyase; polyphenol oxidase; peroxidase; catalase.

Abstract—The stress response of ripening mango fruits treated in the pre-climacteric phase with radiation doses of 0.75, 1.25 and 1.75 kGy was investigated. L-Phenylalanine ammonialyase, polyphenol oxidase, peroxidase and catalase activities were determined and significant differences were observed. Differences in the patterns of total phenolics, flavanols and proteins were also observed. Malic enzyme activity was used as an indicator of the ripening stage (climacteric rise, climacteric peak and post-climacteric phase) as well as a measure of the effect of γ-irradiation on fruit ripening. It seems that radiation treatment causes a stress condition in the fruit which, depending on the dose, may lead to browning of the tissue or necrotic decay.

INTRODUCTION

The γ-radiation-induced delay in ripening processes and senescence in fruits and the accompanying advantages, such as shelf-life extension and disease control, have been reported by several authors for different types of fruit [1]. In a recent communication [2], we showed that irradiation does not cause a true delay in the onset of ripening of fully mature mango fruit, but rather distorts certain biochemical processes so that senescence is delayed.

Depending on the cultivar, the physiological state of the tissue at the time of irradiation, and the dose, some fruits do not tolerate the treatment, and a stress condition arises which can manifest itself in the form of pitting in citrus fruit peel [3] or browning of the skin and flesh tissue in banana [4], mango [5] and avocado [6] fruits. Monitoring the following enzymes, and their substrates and products, viz. phenylalanine ammonialyase (PAL) (EC 4.3.1.5), polyphenol oxidase (PPO) (diphenol:O_2 oxidoreductase, EC 1.10.3.10), peroxidase (POD) (donor: H_2O_2 oxidoreductase, EC 1.11.1.7), catalase (H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6), might give an indication of the extent of stress the γ-irradiated fruit is undergoing, irrespective of the potential outward symptoms [1, 7–10].

The biochemical changes associated with the stress condition induced by γ-irradiation were studied against the background of ripening and senescence of mango fruit. Malic enzyme activity [L-malate: $NADP^+$ oxidoreductase (oxaloacetate decarboxylating), EC 1.1.1.40] was used as an indicator of the climacteric rise, climacteric peak and post-climacteric stage in the fruit.

RESULTS AND DISCUSSION

Identification of the developmental stages of the stressed ripening mango fruit

Malic enzyme is synthesized *de novo* during the climacteric rise which leads to the development of the malate decarboxylating system, and plays an important role in the ripening process of some climacteric fruits [2, 11–13]. Malic enzyme activity normally increases during the climacteric rise, reaching a maximum slightly ahead of the respiration peak (climacteric peak) and then decreases during the post-climacteric phase [2, 14]. Figure 1 shows a similar result for Kent mango fruit as well as a γ-irradiation dose-dependent effect on the enzyme. According to this, the following stages of ripening could be identified, viz. climacteric rise, climacteric peak and post-climacteric phase. The climacteric rise stretches from days 1 to 10 reaching the climacteric peak whereafter the post-climacteric phase follows from the 12th day onward (cf. Fig. 1). The increase in malic enzyme activity, usually observed during the ripening process of mango fruit, was significantly decreased but not delayed by γ-irradiation (Fig. 1). These results are complimentary to those published previously for Haden mangoes where a 0.75 kGy irradiation dose resulted in extensive diminishing of the malic enzyme climacteric peak [2]. According to our results for Kent mangoes, the malic enzyme climacteric peak still exists, but flattens noticeably when increased γ-irradiation is administered (Fig. 1).

PAL activity

PAL is a key enzyme in the metabolism of phenolics; it catalyses the deamination of L-phenylalanine to *trans*-cinnamic acid and thus diverts it from the channels of protein synthesis into those of phenolic synthesis [3]. PAL seems to be extraordinarily sensitive to the physiological state of the plant [10]. Changes in activity can occur during growth, or they may follow traumatic or pathological events or the action of light [15]. Enchanced PAL activity and ethylene evolution in citrus fruits are symptoms of stress conditions, also caused by irradiation [16].

The present report gives evidence for the induction of PAL activity after γ-irradiation, which is dependent on the

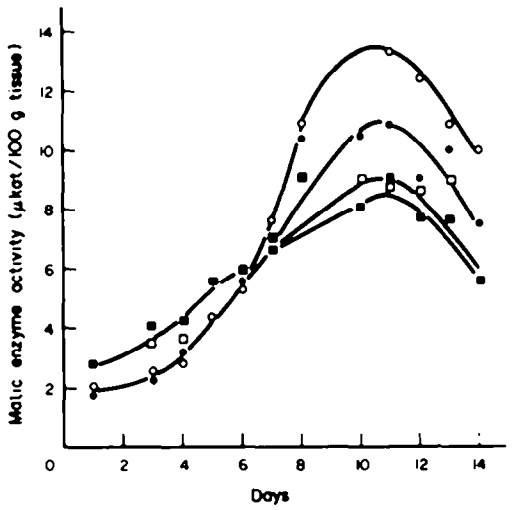

Fig. 1. Changes in malic enzyme activity during ripening in 1.75 kGy (■——■), 1.25 kGy (□——□), 0.75 kGy (●——●) γ-irradiated and control (O——O) Kent mangoes at 20°.

irradiation dose and the duration of storage thereafter (Fig. 2). Within 24 hr after γ-irradiation the 1.75 kGy and 1.25 kGy fruit showed increased PAL activity, reaching a maximum 3–4 days after irradiation (1.75 kGy, 12-fold increase; 1.25 kGy, 8-fold increase; 0.75 kGy, 5-fold increase) compared with a negligible PAL activity peak in the control fruit. After attaining the initial maximum level, the PAL activity declined during the next 3–4 day period. A subsequent second PAL activity peak, similar for all the groups, was reached after the tenth day. The PAL activity declined during the subsequent storage and post-climacteric period. Similar characteristics of the PAL induction process were observed in a number of fruit systems, viz. grapefruit [16] and oranges [3, 9]. This rise in activity is symptomatic of plant tissues subjected to wounding or infection [10, 17] and could be due to an increase in extractibility of the enzyme, caused by irradiation damage to the cell, or it could have been due to activation of a pro-enzyme, or latent forms of the enzyme, if present, or due to some changes caused at the active site of the enzyme [18, 19].

The results obtained support the idea that radiation damage to the tissue in some way triggers the synthesis/activation of PAL.

PPO activity

The specific enzymes that take part in browning reactions involving polyphenols have been known by different names, but in general can be referred to as polyphenoloxidases [8]. In food browning, catecholase action is more important than cresolase action since most of the phenolic substrates in food are dihydroxyphenols [8]. In banana fruits subjected to γ-irradiation, skin browning was observed to be due to activation of PPO [4]. The presence of PPO in mango fruit and changes in this enzyme by γ-irradiation have been shown [5].

Figure 3 shows the relative PPO activity patterns of extracts obtained from mango fruits subjected to different doses of γ-irradiation compared to the control fruit during the ripening and senescence periods. It can be seen that irradiation resulted in a dose-dependent increase in enzyme activity. PPO activity was detected as from the sixth day after irradiation in the 1.75 kGy dose group. The 1.25 kGy group showed PPO activity from the seventh day and the 0.75 kGy group from the tenth day. The control group started showing PPO activity only from the eleventh day, which coincided with the post-climacteric phase. Although highly visible tissue browning was not observed in the Kent cultivar, the changes in the pattern of PPO activation imply a stress response to a condition other than senescence.

Mayer and Harel reported that the strength of binding of latent forms of PPO to membranes appears to vary

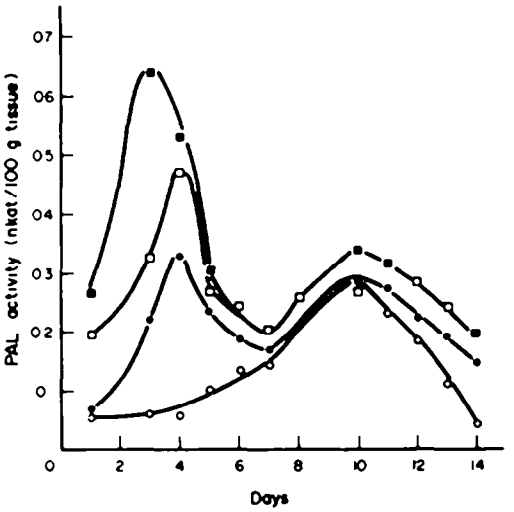

Fig. 2. Changes in PAL enzyme activity during ripening in 1.75 kGy (■- -■), 1.25 kGy (□— —□), 0.75 kGy (●—·—●) γ-irradiated and control (O——O) Kent mangoes at 20°.

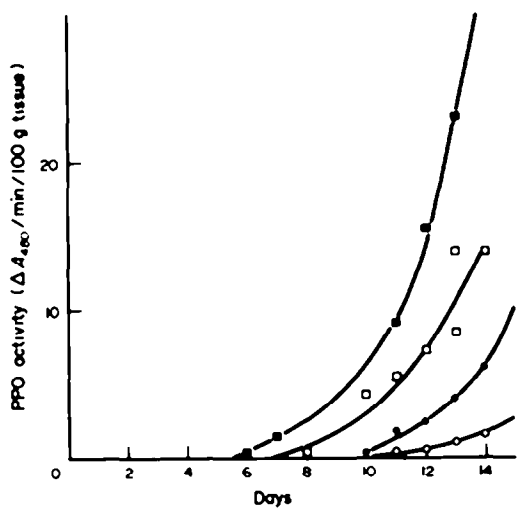

Fig. 3. Changes in PPO enzyme activity during ripening in 1.75 kGy (■——■), 1.25 kGy (□—— □), 0.75 kGy (●——●) γ-irradiated and control (O— —O) Kent mangoes at 20°.

depending on the tissue and the stage of development of the plant [20]. Apparently, conversion of particulate forms of PPO to soluble forms occurs in fruit following exposure to stress conditions [20]. Irradiation causes membrane damage which results in the solubilization of PPO and manifests itself in an earlier rise in activity than in the control group. Solubilization also occurs under more natural conditions, e.g. ripening of fruits or ageing [20]. This explains the rise in PPO activity in the control group at the post-climacteric stage.

POD and catalase activity

POD is known to be involved in the metabolism of phenolic compounds [21] and catalyses the oxidation of a large number of phenols and aromatic compounds which occur naturally in plant tissues [22]. PODs, similarly to PPO, are located in the cell partly in a soluble cytosolic form, and partly in an insoluble, cell-membrane bound form. POD is an inducible enzyme and factors such as physiological stress, wounding, fungal or viral infections are known to bring about changes in POD isoenzyme patterns [7].

The development of POD activity in ripening mango fruit is a specific and orderly process, progressing steadily from the young to the old cell [23, 24], as is reflected in the POD activity pattern of the control fruit (Fig. 4). γ-Irradiation resulted in an almost immediate stimulation of POD activity, which increased steadily during the ripening phase up to the climacteric peak, after which the POD activity seemed to decline. The 1.75 kGy irradiation dose did not increase the POD activity more than the 1.25 kGy irradiation dose and the POD activity patterns overlapped (Fig. 4); it seemed as if the maximum POD activity in the ripening mango fruit was reached at the 1.25 kGy dose. A similar, but relatively lower increase in POD activity was observed for the 0.75 kGy irradiation dose group.

If there is any causal relation between the rise in POD activity and γ-irradiation, one could speculate that it might be mainly related to the elimination of H_2O_2, the production of which in cells increases with increased stress [25]. One of the main products generated during radiolysis of aerobic aqueous solutions is H_2O_2 [26]. Thus an increase in POD activity would represent an induced protective reaction (perhaps delaying senescence and stabilizing fruit decay).

The stimulation by γ-irradiation of mango fruit catalase is somewhat delayed and can be seen from the eleventh day onward, which coincides with the post-climacteric phase of the fruit (Fig. 5). The higher the irradiation dose applied, the higher the stimulation. The substantial increase in the catalase activity towards the beginning of the post-climacteric phase may be attributed to the disorganization of the fruit tissue cells caused by cell membrane damage by γ-irradiation.

The increased supply of oxidizable substrates in intensely respiring tissue, such as ripening fruit [27], is reflected by the increased activities of the oxidative enzymes, viz. peroxidase and catalase [28]. Catalase increased from 1-7 days and thereafter remained constant for control mango fruit (Fig. 5). Irradiation enhanced the peroxidase activity in citrus fruit peel, beginning 1-2 weeks after irradiation [1]. A similar increase in the catalase activity of the external peel layers was observed in irradiated grapefruit, but not in oranges [15].

Total phenolics and flavanols

There have been several reports on the accumulation of phenolic compounds in plant tissues following irradiation

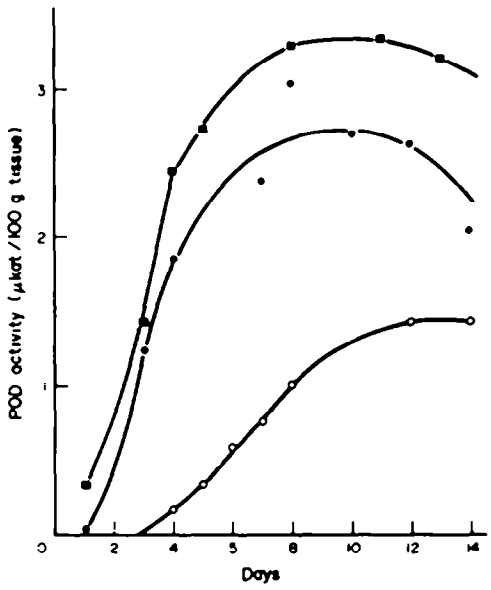

Fig. 4. Changes in POD enzyme activity during ripening in 1.75 kGy and 1.25 kGy (■——■), 0.75 kGy (●——●) γ-irradiated and control (○——○) Kent mangoes at 20°.

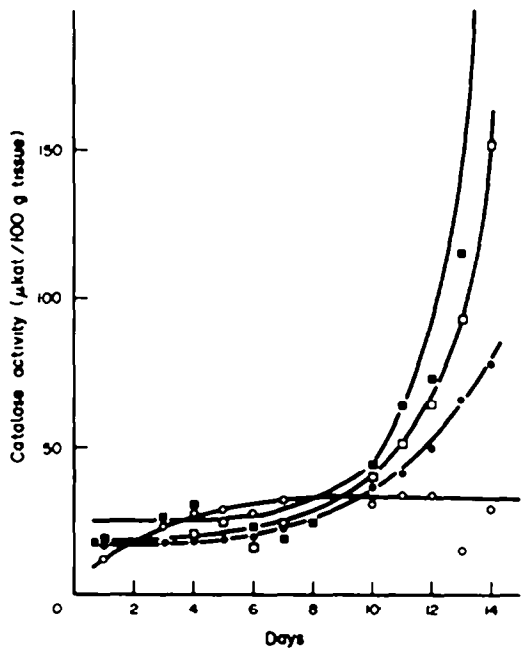

Fig. 5. Changes in catalase enzyme activity during ripening in 1.75 kGy (■——■), 1.25 kGy (□——□), 0.75 kGy (●——●) γ-irradiated and control (○——○) Kent mangoes at 20°.

[3]. It seems that increased phenolic biosynthesis is a typical response of plant tissues to irradiation, reflecting a stress condition [3].

There was no clear additional accumulation of phenols after the PAL activity peak had been attained (Fig. 6). This may be due to the removal of phenols by some mechanism like oxidation of phenols to quinones, which are not reactive to the assay for phenolic compounds [29]. Such oxidation may be carried out by PPO, which has been shown to be enhanced as an effect of irradiation (Fig. 3). Similar results were obtained for citrus fruits [9].

On examining methanolic extracts from the irradiated fruit tissues as compared to the control fruits, a marked accumulation of flavanols was observed (Fig. 7).

Proteins

There seems to be no significant difference between the total soluble protein content pattern during the ripening and senescence periods of the irradiated groups in comparison to the control fruit. The protein content seems to increase during the ripening phase until it reaches the climacteric peak whereafter it stabilizes and may even decrease during the post-climacteric stage. In the case of grapefruit peel, a linear relationship between the increase in irradiation dose and the inhibition of protein synthesis has been found [30]. This is in contrast to our finding.

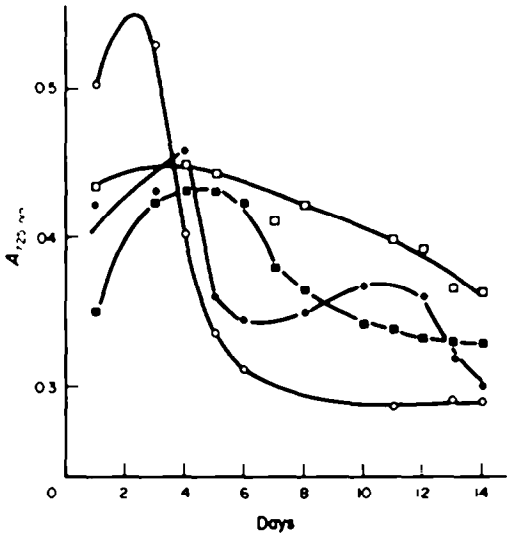

Fig. 6. Changes in the total phenolic concentration during stressed ripening in 1.75 kGy (■- -■), 1.25 kGy (□- -□), 0.75 kGy (●- -●) γ-irradiated and control (○—○) Kent mangoes at 20°.

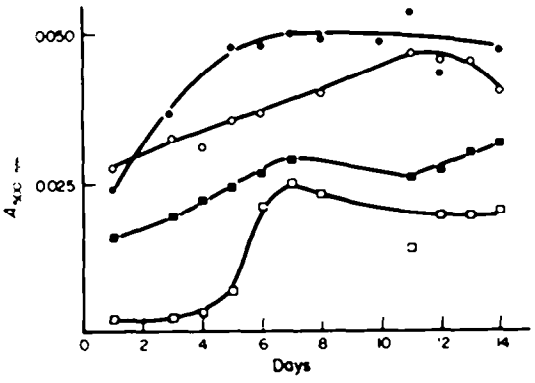

Fig. 7. Changes in the flavanol content during stressed ripening in 1.75 kGy (■ ---■), 1.25 kGy (□- -□), 0.75 kGy (●——●) γ-irradiated and control (○- -○) Kent mangoes at 20°.

CONCLUSIONS

The physiological effects of γ-irradiation may be immediate or somewhat delayed [31]. During this study, we observed an immediate increase in PAL and POD activity soon after irradiation. In contrast, the enhancement of PPO and catalase activity in Kent mango fruit appears to be a delayed effect of γ-irradiation similar to that reported for the activation of PPO [5] and catalase [32] of flavedo of citrus fruit.

The activation of PPO in mango fruit upon irradiation could be caused in many ways. It can be due to an activation of a pro-enzyme or a latent enzyme or due to conformational changes in the enzyme [20]. It is a special characteristic of many, if not all, plant tissues that phenolic substances, which act as substrates for PPO and POD, are either sequestered in special cells or in the vacuole, away from the enzymes. When there is any mechanical or physiological injury (e.g. by γ-irradiation), these substrates and enzymes come into contact with each other, and in the presence of atmospheric oxygen rapid browning could occur [8]. The strength of binding of PPO and POD to membranes appears to vary depending on the tissue and the stage of development of the plant. Solubilization occurs under more 'natural' conditions, e.g. ripening of fruits or ageing. Thus, PPO and POD become increasingly soluble during fruit ripening [11, 20]. Consequently, they become more and more extractable and this could be the reason why a higher activity was detected in the post-climacteric stage of the control fruit. Solubilization could also occur as a result of membrane damage caused by γ-irradiation.

The results obtained indicate that radiation treatment of mango fruits leads to a stress condition which, depending on the dose, can be overcome or can lead to a gross distortion of normal biochemical patterns during the phases of ripening and senescence.

EXPERIMENTAL

Source of fruits. Mature, fully developed Kent mangoes (*Mangifera indica* L.), were obtained from orchards in the Tzaneen area. Fruit were selected for the expts according to similarity in size, mass, ripening phase (pre-climacteric) and absence of disease.

Radiation treatment. Irradiation was done in a gamma beam 650 (AECL) irradiator equipped with a ^{60}Co source at a dose rate of *ca* 4 kGy/hr. Dosimetry was performed using a Fricke dosimeter [33]. Irradiation was performed in air and at room temp. The fruits were divided into three groups treated with doses of 0.75 (used for shelf-life extension of South African fibreless cultivars) 1.25 and 1.75 kGy, respectively.

Storage and sampling techniques. The same set of expts was repeated on mango fruit over a few seasons and similar results were obtained. The control and irradiated groups contained 70 fruits each and were kept in an air-conditioned room at 20°. The

sampling was conducted over a period of 14 days, 6–8 fruits per group were peeled, immediately shredded, mixed thoroughly and frozen with liquid N_2. The frozen tissues were stored at −20° until further use.

Extraction and assay of PPO and POD activity. Enzyme extraction was based on the method described by Van Lelyveld *et al.* [34]. 7.5 g of fruit tissue, 1.5 g of polyethylene glycol 4000 and 45 ml Me_2CO (−20°) were homogenized with a Sorval Omni-mixer for 60 sec. The homogenate was filtered through a sintered-glass filter (G3) and washed with a further 50 ml Me_2CO (−20°). The Me_2CO powder was homogenized for 2 min with 15 ml (4°) 10 mM OAc buffer (pH 5.0) and centrifuged at 10000 *g* for 30 min at 0°. The supernatant soln was treated with a further two vols. of Me_2CO (−20°) and centrifuged at 20000 *g* for 30 min at 0°. The ppt. was suspended in 7.5 ml (4°) 10 mM OAc buffer (pH 5.0) and centrifuged at 20000 *g* for 30 min at 0°. The resulting supernatant was then used for POD and PPO enzyme assay.

The POD reaction mixture consisted of 20 ml 20 mM OAc buffer (pH 5.0) to which was added 1 ml 100 mM H_2O_2 and 1 ml 40 mM guaiacol [27]. 0.1 ml enzyme extract was added to 4 ml of the reaction mixture and incubated for 2 min at 30° whereafter POD activity was determined by measuring the rate of change in absorption at 420 nm and 30°. Calculations were made from the initial steepest portion of increase in the absorbance curve. One unit of enzyme activity (1 Kat) was defined as the amount of enzyme that catalyses the consumption of 1 mol of H_2O_2 and 1 mol of guaiacol per sec.

PPO activity was determined by incubating 10 ml 10 mM OAc buffer to which was added 0.1 ml 0.13% $CuSO_4$ and 2 ml of the same buffer containing 0.02 g catechol. Immediately after adding 1 ml of enzyme extract to 4 ml of the above-mentioned reaction mixture, the rate of browning was recorded at $A_{480\,nm}$ and 30°. PPO activity was expressed in terms of $\Delta A_{480\,nm}$/min.

Extraction and assay of PAL activity. An Me_2CO powder prepared as described above for the extraction and assay of PPO and PO was homogenized for 2 min with 15 ml (4°) 10 mM borate buffer (pH 9.2) containing 1 mM DTT and 0.1 mM mercaptobenzotiazole and centrifuged at 20000 *g* for 30 min at 0°. The supernatant soln was used for the PAL assay. The PAL reaction mixture consisted of 100 mM borate buffer (pH 8.9) containing 1 mM DTT, 0.1 mercaptobenzotiazole and 10 mM L-phenylalanine incubated at 40°. 0.5 ml enzyme extract was added to 2.5 ml of the reaction mixture and incubated for 2 hr at 40° whereafter the absorbance was determined at 270 nm. A control was determined by reading the absorbance of the above-mentioned soln immediately without incubation. By subtracting the absorbance reading of the control from the absorbance reading of the reaction mixture incubated for 2 hr, the PAL activity was determined as $\Delta A_{270\,nm}$/2 hr. One unit of PAL enzyme activity (1 Kat) was defined as the amount of PAL enzyme that catalyses the conversion of 1 mol of L-phenylalanine per sec under the assay conditions.

Extraction procedure for malic enzyme and catalase. The procedure described by Dubery *et al.* [35] for malic enzyme was applied. Mango tissue (15 g) was homogenized with 30 ml 100 mM Tris HCl buffer (pH 8) for 1 min at 4° in the presence of 0.3 g Polyclar AT. The homogenate was adjusted to pH 7.1 and then centrifuged at 15000 *g* for 10 min at 4°. The supernatant was used for determining the malic enzyme and catalase activity.

Catalase was assayed by recording the change in absorbance at 240 nm. The reaction mixture (3 ml) contained 0.3% H_2O_2 in 0.05 M Na-Pi buffer (pH 6.5) and 0.5 ml enzyme extract [6]. One unit of enzyme activity (1 Kat) was defined as the amount of catalase that catalyses the conversion of 1 mol of H_2O_2 per sec.

Malic enzyme activity was monitored as described by Dubery *et al.* [35] by measuring the reduction of NADP at 340 nm. The reaction mixture contained 10 mM Tris–HCl buffer at the optimum pH of 7.1, 1 mM $MnSO_4$, 5 mM L-(−)-malate, 0.5 mM NADP and enzyme in a total vol. of 3 ml [36]. The reaction was initiated by the addition of enzyme and under these conditions the relation between reaction rate and enzyme concn was linear. The temp. of the cell compartment was thermostatically controlled at 30°. One unit of enzyme activity (1 Kat) was defined as the amount of enzyme that catalyses the conversion of 1 mol of L-(−)-malate to pyruvate and CO_2 per sec.

Total phenols and flavanols. 5 g mango tissue (instantly frozen with N_2 and stored at −20°) was homogenized with 40 ml 70% MeOH in a Sorvall Omni-mixer for 5 min and then centrifuged at 20000 *g* for 10 min. Total phenol and flavanol concns were determined by the methods described by Swain and Hilles [29].

Total soluble protein content for each extract was measured by the method of Lowry *et al.* [37] with bovine albumin as standard.

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