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Changes in the Activities of Catalase, Peroxidase, and Polyphenol Oxidase in Apple Buds During Bud Break Induced by Thidiazuron

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Abstract. The breaking of dormancy in apple buds (*Malus domestica* Borkh cv. York Imperial) by thidiazuron (*N*-phenyl-*N'*-1,2,3,-thidiazol-5-ylurea) was investigated in relation to catalase, peroxidase, and polyphenol oxidase activities and their isoenzyme patterns. The activity and number of isoenzymic components of catalase increased progressively during bud break, then decreased after buds started to grow. Peroxidase activity was highest during dormancy and declined during bud swell, increased at bud break, and decreased after bud expansion. Several isoperoxidases were observed in gel electrophoresis. Similar patterns were found at different growth stages of apple buds except for one peroxidase isoenzyme, P3, which disappeared 12 days after thidiazuron treatment. There was an inverse relationship between the activities of polyphenol oxidase and peroxidase during the development of apple buds. Apple buds have a very similar polyphenol oxidase isoenzyme pattern throughout bud development. However, the appearance and disappearance of minor isoenzymes were also observed. Phloridzin, rutin, p-coumaric, epicatechin, naringin, chlorogenic acid, and catechol were found in apple buds. Among them, phloridzin, rutin, and p-coumaric were the dominant phenolic compounds. Dormant buds contained a high amount of phenolic substances which decreased after bud break (4 days after thidiazuron treatment) then increased until the start of bud expansion. Phenolic compounds are found to be potent modifiers of catalase, peroxidase, and polyphenol oxidase activity, as both inhibitors and stimulators in apple buds.

Apple buds require a certain amount of exposure to low temperatures before resumption of growth in spring. This cold requirement can be overcome by treating the buds with certain plant hormones and growth regulators. We previously reported that the plant bioregulant thidiazuron (*N*-phenyl-*N'*-1,2,3-thidiazol-5-ylurea) has the capacity to release apple lateral buds from dormancy and to induce metabolic changes (Wang and Faust 1988a, Wang et al. 1986, 1987). A number of studies demonstrated that the physiological alterations produced in plants due to different causes are related to enzymes that catalyze oxidative reactions. During growth and development, changes in isoenzymes are often dramatic. The appearance of new electrophoretic bands and the disappearance of others may relate to the "turning on" or "derepression" of genes that control or modify the synthesis of enzymes (Beckman et al. 1964, Scandalios 1969, Upadhyaya and Yee 1968). Some investigations (Pollock 1953, Thornton 1965) have shown that the amount of O_2 in buds is the main factor in regulating the biochemical transformation leading to rest or termination of rest.

Catalase, peroxidase, and polyphenol oxidase are all oxidative enzymes. The objective of this study was to determine the changes of these enzymes during dormancy, bud break, and bud growth in apple buds in response to thidiazuron treatment. Furthermore, phenolic substances are common in plants, and studies have shown that they are physiologically active and influence many enzyme systems as both inhibitors and stimulators. Therefore, the aim of this work was also to establish the chemical nature of phenols present in apple buds and to determine their effect on catalase, peroxidase, and polyphenol oxidase.

Materials and Methods

Plant Materials and Treatments

The growing conditions and treatments of apple seedlings (*Malus domestica* Borkh cv. York Imperial) used in this study have been

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previously reported (Wang et al. 1986). The seedlings were planted in 8 inch pots, generally 0.5 m high with a single stem and at least 15 nodes, and grown in a greenhouse; they had not received any chilling. The plants were randomized and a total of 1350 seedlings were used for the experiment. Thidiazuron (*N*-phenyl-*N'*-1,2,3-thidiazol-5-ylurea) (100 μM) was dissolved in 2.5% (vol/vol) DMSO plus 0.5% (vol/vol) Tween-20 and applied directly with a brush to the top five lateral buds (the leaves were still on the plant) until runoff. Control plants were treated with DMSO and Tween-20 only. The natural daylight in the greenhouse was supplemented with 400-W high-pressure sodium lamps (Energy Technics, York, PA, USA), which provided a PAR level of about 320–400 μmol $m^{-2}s^{-1}$ for 10 h per day (7:00 a.m. to 5:00 p.m.). Temperatures were approximately 25°C during the day and 20°C at night, and the relative humidity was 65%. Buds from control and thidiazuron-treated plants were sampled at 2-day intervals during a 12-day period. Triplicate bud samples of 0.1-g fresh weight were collected at each sampling time for each enzyme assay. Depending on their size, the number of buds required ranged from approximately 6 to 102. Forty buds were used for fresh weight and dry weight determination.

Enzyme Extraction and Assay

Catalase. Bud tissue was pulverized with a cold mortar and pestle in a Tris-HCl buffer (pH 8.5) (1 ml of buffer per 100 mg of tissue) containing 5 mM EDTA-Na, 5 mM DTT, 10% (wt/vol) insoluble polyvinyl pyrrolidone (PVP), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (PMSF added just before extraction). The homogenate was strained through four layers of miracloth and centrifuged at 20,000 *g* for 30 min at 4°C. The supernatant was used for assays of catalase activity. The catalase activity was determined by the floating disc method (Nir et al. 1986). Discs 6 mm in diameter, were cut from Whatman 3 MM chromatographic paper, and 10 μl of crude enzyme extract was applied to each disc and placed in a vial containing 5 ml of 30 mM H_2O_2 at 20°C. The elapsed time for the discs to float was determined with a stopwatch. Ten to 20 replicates of individual discs were used for each crude extract. The activity of catalase in the apple bud extracts was calculated according to the activity of bovine-liver catalase (σ = 11,000 U/mg protein). One unit of catalase activity was expressed as micromoles of H_2O_2 degraded per min at 20°C.

Peroxidase. Fresh bud tissue (100 mg) was homogenized with a chilled mortar and pestle in 4 ml cold 0.1 M phosphate buffer (pH 6.1) containing 30 mg of insoluble PVP and 15 mg sodium ascorbate. The homogenate was filtered through four layers of miracloth and centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was used for the peroxidase assay. The assay mixture contained 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol as donor, 3 mM H_2O_2 as substrate, and 0.4 ml crude enzyme extract. The total reaction volume was 1.2 ml. The rate of change in absorbance (OD) at 420 nm was measured by a spectrophotometer (Shimadzu UV-160A). The levels of enzyme activity were expressed as the OD difference per minute per milligram protein.

Polyphenol Oxidase. Fresh bud tissue (100 mg) was rapidly ground with a chilled mortar and pestle in 1 ml of cold acetate buffer (0.01 M, pH 6.0). The homogenate was passed through four layers of miracloth and centrifuged at 12,000 *g* for 15 min at 2°C. The supernatant was used for the polyphenol oxidase assay. The reaction mixture contained 2.70 ml of 0.01 M acetate buffer (pH 6.0), 0.25 ml of 0.5 M catechol, and 50 μl of crude enzyme extract. The activity was measured by monitoring O_2 (YSI model 53) and was expressed as nanomoles of O_2 consumed per minute per microgram protein.

Protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard. The effect of 4-chlororesorcinol and phenolic compounds on the activity of the above three enzymes was also determined. Test compounds at different concentrations were preincubated with crude extracts of catalase, peroxidase, or polyphenol oxidase and their defined reaction medium for 5 min before substrates were added. The measurement of individual enzyme activity was as described above.

Electrophoresis

Crude enzyme extracts were prepared as described above. The extraction buffer was 0.1 M Tris-HCl (pH 8.0) with the following composition: 0.6 M sucrose, 5 mM sodium ascorbate, 5 mM cysteine-HCl, and 10 mM EDTA-Na. Polyacrylamide gels were prepared for isozyme analysis. Five percent gels (containing 0.5% soluble starch) were used for catalase and 7% gels for peroxidase and polyphenol oxidase isozyme separations. The stacking gel was 2.5% for all enzymes. Tris-HCl buffer (1.0 M, pH 8.9) was used to prepare the gels. Tris-glycine (0.005 M, pH 8.3) buffer was the electrode buffer. The initial current for electrophoresis was 20 mA. After the bromophenol blue dye front entered the separating gel, the current was increased to 30 mA. Catalase isozyme bands were visualized by directly staining the gel with a solution containing 60 mM Na-thiosulfate and 3% H_2O_2 (freshly mixed before use) for 30 s, then transferring to 90 mM potassium iodide and 5% acetic acid. Achromatic bands appeared in gels (bands diffused after a period of time in air). Peroxidase bands were visualized by staining directly with benzidine (0.15% benzidine solution contained 2.5 mM H_2O_2, 10 mM EDTA-Na, and 0.75 M NH_4Cl). Polyphenol oxidase bands were visualized by staining with 5 mM DL-β-(3,4-dihydroxyphenyl) alanine (DOPA) in 0.1 M acetate buffer (pH 6.0) under aeration.

Molecular weights of the major isoenzymes of catalase, peroxidase, and polyphenol oxidase were determined indirectly as described for nondenatured protein (Sigma 1986). The protein (isoenzyme) was electrophoresed on a set of gels of various polyacrylamide concentrations and the Rf of the protein in each gel was determined relative to the tracking dye; 100 Log (Rf × 100) was plotted against the percent gel concentration for each protein, where Rf is the electrophorectic mobility. The slope of such a plot is the retardation coefficient (K_R). From these plots, individual slopes (K_R) were determined for each protein and the logarithm of the negative slope was plotted against the logarithm of the molecular weight of each protein. This produced a linear plot from which the molecular weight of each isoenzyme was determined. α-Lactalbumin, carbonic anhydrase, chicken egg albumin, bovine serum monomer albumin, bovine serum dimer albumin, Jack bean trimer urease, and Jack bean hexamer (molecular weights: 14.2, 29, 45, 66, 132, 272, and 545.0 (kD), respectively) were used as standard proteins.

Determination of Phenolics in Apple Buds

Extraction, fractionation, and high-performance liquid chromatography (HPLC) analyses of phenolics were performed follow-

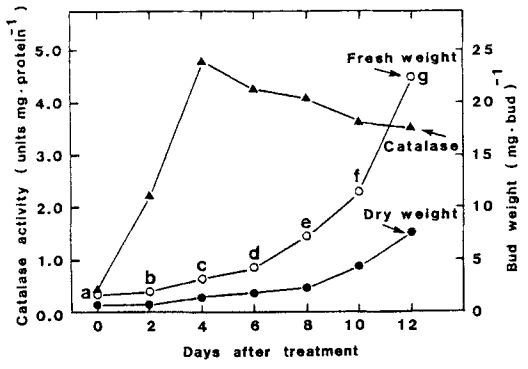

Fig. 1. Changes in apple bud catalase activity, fresh and dry weights associated with dormancy, bud break, and resumption of growth induced by 100 μM thidiazuron are shown. Stages of bud development were classified as follows: stage a, dormant bud; stage b, first swell; stage c, full swell; stage d, side green; stage e, green tip; stage f, bud expansion start; stage g, bud open. Letters inside figure indicate stages of development. LSD (5%) for catalase activity, 0.45; fresh weight, 1.25; dry weight, 0.61.

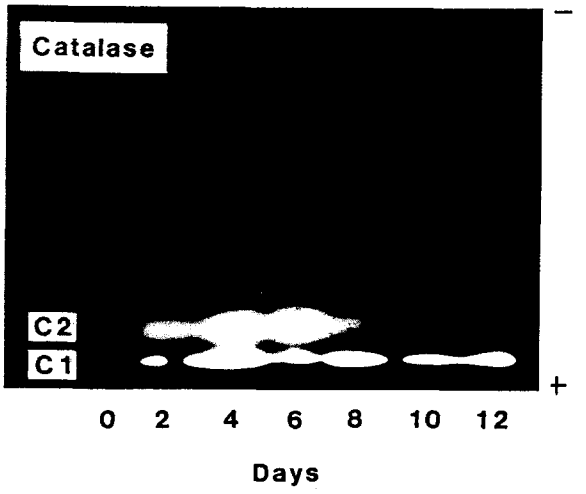

Fig. 2. Changes in apple bud catalase isoenzyme patterns associated with dormancy, bud break, and resumption of growth induced by thidiazuron are shown.

ing the procedure described by Coseteng and Lee (1987). Total phenolics were measured by Folin and Ciocalteu phenol reagent (Sigma Chemical Co., St. Louis, MO, USA). Separation of phenolics was performed on an 8 mm × 10 cm C18 Radial-PAK column (Waters Associates, Millipore Corp., Milford, MA, USA) at room temperature, and spectrophotometric detection was performed at 280 nm. The column was conditioned with solvent A (5% acetic acid in water), and the phenolics were eluted using a linear solvent gradient of 0–100% solvent B (40% acetonitrile in water) for 1 h at a flow rate of 1 ml/min. Individual phenolic compounds were identified by comparison with authentic standards.

Results and Discussion

Catalase, Peroxidase, and Polyphenol Oxidase

The control buds remained in dormancy throughout the experiment. There were no significant changes in fresh weight, dry weight, or the activities and isoenzymic patterns of catalase, peroxidase, and polyphenol oxidase (data not shown).

Changes in apple bud fresh weight, dry weight, and catalase activity during dormancy and at different developmental stages after thidiazuron treatment are shown in Fig. 1. An increase in fresh and dry weights was associated with bud break and bud growth induced by thidiazuron. Dormant buds have a low catalase activity. Low levels of catalase activity may result in increased levels of H_2O_2 in bud tissue. Increased catalase activity was associated with bud break. The catalase activity reached its peak when the buds were in the early stage of swelling 4 days after treatment, and slightly declined thereafter. A similar phenomenon was also found in peach flower buds as dormancy was broken and growth began (Kaminski and Rom 1974). It has been reported that catalase functions in ensuring the removal of H_2O_2, supplying free O_2, and detoxifying harmful metabolic products (Burris 1960). The increase in free O_2 supply has been proposed to be the "trigger mechanism" in bud break (Kaminski and Rom 1974). The isoenzymic patterns of catalase in thidiazuron-treated apple buds are shown in Fig. 2. The increase in intensity of the catalase bands was associated with the catalase activity level during early bud break (Figs. 1 and 2). Activation of catalase isoenzymes C1 and C2 coincided with bud break. The molecular weights of isoenzymes C1 and C2 were estimated to be 53.2 and 59.6 kD, respectively. Isoenzyme band C2 disappeared after the start of bud expansion (Fig. 2), this indicates that apple buds possess a specific catalase isoenzymic pattern at a particular stage of thidiazuron-induced growth. Increases in multimolecular forms of catalase during bud break may be a result of de novo protein synthesis.

Peroxidases are involved in a large number of biochemical and physiological processes (Yip 1964) and may change quantitatively and qualitatively during growth and development (Shannon 1969). Alterations in both the activity and isoenzyme pattern of peroxidase by hormones, such as indole-3-acetic acid (IAA) and gibberellic acid, have been reported previously (Galston et al. 1968, Halevy 1963). The level of peroxidase activity in dormant buds was relatively high, then decreased during early stages of bud break, and increased again during early bud growth (Fig. 3). High peroxidase activity in dormant buds may have resulted from the inhibition of catalase. The mode of action of perox-

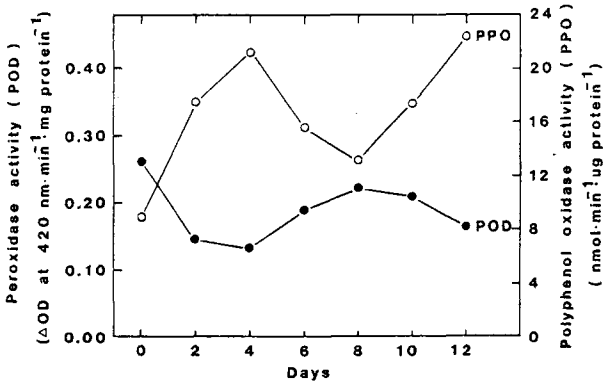

Fig. 3. Changes in the activities of apple bud peroxidase and polyphenol oxidase associated with dormancy, bud break, and resumption of growth induced by thidiazuron. LSD (5%) for peroxidase, 0.04; polyphenol oxidase, 1.89.

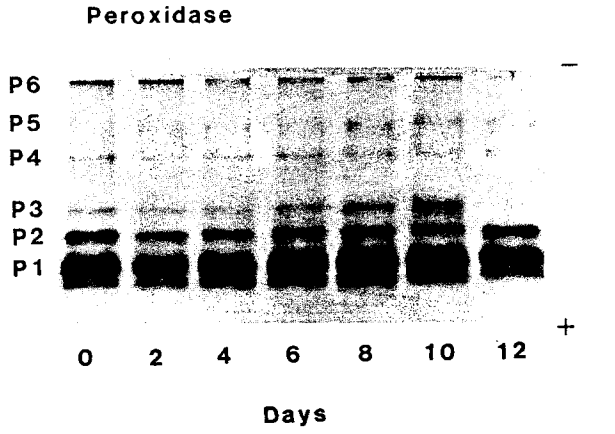

Fig. 4. Changes in apple bud peroxidase isoenzyme patterns associated with dormancy, bud break, and resumption of growth induced by thidiazuron are shown.

idase on the H_2O_2 substrate differs from catalase action in that peroxidase liberates free radicals rather than oxygen. These free radicals are highly phytotoxic. Wang and Faust (1988b) demonstrated that free radicals are formed in dormant buds, and that thidiazuron induced bud break while diminishing free radical formation. The accumulation of H_2O_2 may cause changes in plant metabolism. H_2O_2 may oxidize sulfhydryl groups and inactivate IAA (Omran 1977, Stonier and Yang 1973). However, IAA inactivation can be reversed upon the introduction of catalase. The H_2O_2 formed by peroxidase may be scavenged by catalase. In the present work, when the buds were dormant, the peroxidase level was high, and the catalase activity was low. However, when buds were treated with thidiazuron, catalase activity increased and peroxidase activity decreased within 2 days, concomitant with bud swell (Figs. 1 and 3). After bud swell, peroxidase activity increased and then decreased when buds started to expand and bud fresh and dry weights showed rapid increases (Figs. 1 and 3). These results indicate that peroxidase may have a significant role in the regulation of cell growth and differentiation during bud break.

Multiple molecular forms of peroxidase have been found in extracts of many plant tissues (Shannon 1968). Biochemical studies comparing activities of isolated peroxidases using various hydrogen donors suggest that these enzymes may have several different functions in plants (Burris 1960, Shannon 1968). The isoenzyme pattern of peroxidase in apple buds was composed of many bands with significant, but not dramatic, changes during bud break. Six bands (isoenzymes) of the peroxidase enzyme were observed in apple buds at different stages of development when benzidine was used as the electron donor for peroxidase (Fig. 4). Variation was observed with respect to the number and intensity of isoenzymes. However, a correlation was found for the intensity, number of isoenzymes, and activity of the enzyme (Figs. 3 and 4). The molecular weights of peroxidase isoenzymes P1, P2, and P3 were estimated to be 58.1, 67.0, and 80.5 kD, respectively. Isoenzyme P1 showed the strongest enzymic activity, while P4, P5, and P6 were only present in trace amounts. Isoenzyme P3 was present at all stages of bud development except 12 days after induction of growth. The disappearance of the isoenzyme P3 at 12 days after thidiazuron treatment may indicate that the gene(s) responsible for P3 production may be inhibited at the time of bud expansion.

Multiple forms of polyphenol oxidase are known to exist in various plants and many catalytic functions have been ascribed to the enzyme. Each isoenzyme is thought to perform a particular physiological action at a particular phase of development (Beckman and Johnson 1964). Apple buds have three polyphenol oxidase isoenzymes, PP1, PP2, and PP3, and their patterns are very similar during all stages of bud development (Fig. 5). However, the appearance and disappearance of minor isoenzymes were observed during dormancy, bud break, and bud growth. The molecular weights of PP1, PP2, and PP3 were estimated to be 32.3, 39.1, and 76.8 kD, respectively. Isoenzyme PP1 showed the strongest enzymic activity. By using DL-β-(3,4-dihydroxyphenyl) alanine (DOPA) to detect polyphenol oxidases and benzidine to locate peroxidase on polyacrylamide gels after electrophoresis, similar band patterns for these two enzymes in apple buds were seen (Figs. 4 and 5). Several researchers have speculated that polyphenol oxidase isozymes

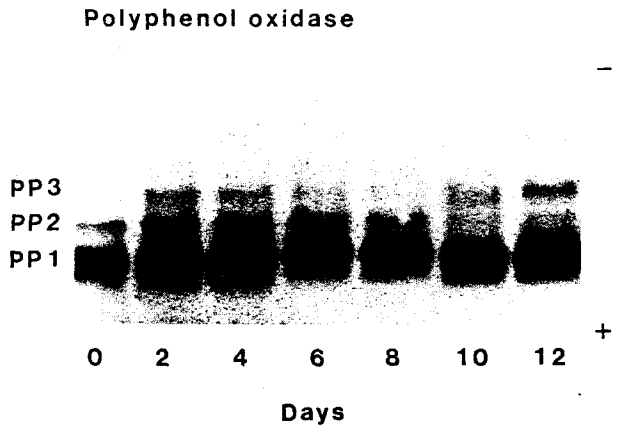

Fig. 5. Changes in apple bud polyphenol oxidase isoenzyme patterns associated with dormancy, bud break, and resumption of growth induced by thidiazuron are shown.

also exhibit peroxidase activity (Srivastava and Van Huystee 1973). A similarity between zymograms of these enzymes developed from the extracts of tobacco leaves has been reported (Sheen and Calvert, 1969). However, these enzymes have independent activities in apple buds (Fig. 3). They also have different responses to 4-chlororesorcinol. As a polyphenol oxidase inhibitor (Gad and Ben-Efraim 1988), 4-chlororesorcinol inhibited polyphenol oxidase activity in apple buds, but showed no effect on apple bud peroxidase activity (Table 1). There is an inverse relationship between the activities of peroxidase and polyphenol oxidase in apple buds during dormancy, bud break, and bud development (Fig. 3). Dormant apple buds have low polyphenol oxidase activity, but high peroxidase activity. Two days after thidiazuron treatment, buds started to swell and polyphenol oxidase activity increased while peroxidase activity decreased. Polyphenol oxidase decreased and peroxidase increased again when buds approached the greentip stage, and then their activities reversed when buds started to expand 10 days after thidiazuron treatment. Thus, changes in enzyme patterns with growth may reflect the repression and derepression of specific genes which produce the enzymes and structural proteins characteristic of each tissue during bud development.

Phenolic Compounds

Phloridzin, rutin, p-coumaric, epicatechin, naringin, chlorogenic acid, and catechol were found in apple buds (Fig. 6). Phloridzin, rutin, and p-coumaric were the dominant phenolic compounds.

Table 1. Effect of 4-chlororesorcinol on activities of peroxidase and polyphenol oxidase of apple buds.

Inhibitor concentration (μM)	Peroxidase (% of control)	Polyphenol oxidase (% of control)
5.0	100.0	57.58
0.5	100.0	68.69
0.05	100.0	86.37
0.005	100.0	96.47 NS

Samples were taken 6 days after 100 μM thidiazuron treatment. Significant at 5% level as compared to the control. NS, nonsignificant.

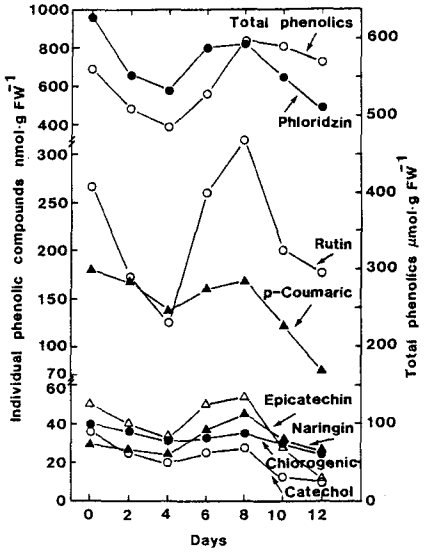

Fig. 6. Changes in individual phenolic compounds in apple buds associated with dormancy, bud break, and resumption of growth induced by thidiazuron are shown. LSD (5%) for total phenolics, 24.7; phloridzin, 76.5; rutin, 41.8; p-coumaric acid, 27.6; epicatechin, 12.5; naringin, 5.3; chlorogenic acid, 4.3; catechol, 12.4.

Phloridzin occurs in many *Malus* species (Harborne and Simmonds 1964) and is the dominant polyphenol in apple buds. Dormant buds contained high amounts of total phenolics, phloridzin, rutin, p-coumaric, and epicatechin (Fig. 6). The biosynthesis of phenolics may exceed the rate of use during dormancy. Phenolic compounds decreased during the first 4 days after thidiazuron treatment, then rapidly increased until bud expansion, and finally decreased again. These results suggest that the phenolic compounds in apple buds are metabolized or translocated to other parts of the plant to meet development needs. Phenols are potent modifiers of enzyme activity as both inhibitors and stimulators, and could be one of the transducers of physiological stimuli that play an important role in connecting the

Table 2. Effect of phenolic compounds on catalase, peroxidase, and polyphenol oxidase activities in apple buds.

Phenolic compound	Concentration (μM)	Catalase[a]	Peroxidase[a]	Polyphenol oxidase[a]
Catechol	1	87.9	90.3	—
	10	77.6	85.7	—
	100	67.3	69.4	—
Catechin	1	100.6 NS	103.7 NS	103.3 NS
	10	98.2 NS	127.6	94.7 NS
	100	88.0 NS	180.9	88.5 NS
Epicatechin	1	106.1 NS	156.2	100.0 NS
	10	100.0 NS	164.7	97.9 NS
	100	102.0 NS	211.3	96.4 NS
4-Methylcatechol	1	101.2 NS	112.3	100.0
	10	98.2 NS	104.8 NS	87.9
	100	69.7	21.0	86.3
Naringin	1	98.2 NS	55.3	96.1 NS
	10	86.1	48.6	92.9 NS
	100	75.8	47.5	92.9 NS
Rutin	1	101.8 NS	50.5	81.2
	10	102.4 NS	41.9	71.7
	100	92.7 NS	38.2	62.5
Phloridzin	1	98.8 NS	56.5	104.1 NS
	10	91.5	50.5	94.2 NS
	100	82.4	42.8	83.7
Chlorogenic acid	1	100.0	52.3	98.1 NS
	10	101.8 NS	52.3	102.4 NS
	100	101.8 NS	51.4	99.3 NS

Samples were taken 4 days after 100 μM thidiazuron treatment. Significant at 5% level as compared to the control. NS, nonsignificant.
[a] Percent activity of control.

environment with the biology of the organism. The effects of phenolic compounds, catechol, catechin, epicatechin, 4-methylcatechol, naringin, rutin, phloridzin, and chlorogenic acid, on catalase, peroxidase, and polyphenol oxidase activities are shown in Table 2. Catechol, 4-methylcatechol, naringin, and phloridzin at 100 μM inhibited catalase activity. Lower concentrations had less or no inhibitory effect (Table 2). Catechol, 4-methylcatechol, naringin, rutin, phloridzin, and chlorogenic acid inhibited peroxidase activity. However, catechin and epicatechin stimulated peroxidase activity (Table 2). Many phenolic compounds are oxidized by polyphenol oxidase, but some of them do not serve as substrates for these enzymes. Furthermore, many phenolic compounds can either inhibit or activate polyphenol oxidase from several plant sources. High concentrations (100 μM) of rutin, phloridzin, and 4-methylcatechol inhibit the oxidation of catechol by apple bud polyphenol oxidase, and the inhibitory strength decreased with decreasing concentration of phenolic compounds (Table 2). Furthermore, the peak rutin concentrations coincided with the lowest polyphenol oxidase activities (Figs. 3 and 6); rutin was also the strongest in vitro inhibitor of polyphenol oxidase activity.

Conclusions

The activities and isoenzyme patterns of apple bud (catalase, peroxidase, and polyphenol oxidase) were affected by thidiazuron treatment, which also induced bud break. The activity of catalase isoenzymes increased progressively with bud break induced by thidiazuron, then declined slightly thereafter. Peroxidase activity decreased at bud break, but increased after bud swell. When buds started to expand, peroxidase decreased again. There was an inverse relationship between the activities of polyphenol oxidase and peroxidase in apple buds during their development. Multiple molecular forms of isoenzymes have been found in apple buds during dormancy and bud break. The appearance or disappearance of a particular isoenzymic component at a particular stage of growth and development of apple buds may be dependent upon the metabolic requirements of the bud at that particular stage of growth. The isoenzyme differences at various developmental stages may also be the result of the activity of structural and regulatory genes controlling the developmental state of the apple buds. Phenolic compounds, phloridzin, rutin, p-coumaric, epicatechin, naringin, chlorogenic acid, and catechol, were

found in apple buds, and these are potent modifiers of the activities of enzymes, catalase, peroxidase, and polyphenol oxidase, as both inhibitors and stimulators, and may influence growth processes in apple buds. Whether the thidiazuron-induced changes in these sequential events in buds are similar to those occurring in buds grown under natural conditions during dormancy and bud break warrants further study.

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