Activities of Catalase and Pentose Phosphate Pathway Dehydrogenases during Dormancy Release in Nectarine Seed

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additional index words. glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, *Prunus persica*

Abstract. The activities of catalase and of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), the two key enzymes in the pentose phosphate pathway (ppp), were measured in the seeds of *Prunus persica* (L.) Batsch var. *nectarina* Maxim 'Nectarine 7'. The seeds were subjected to three imbibition treatments: 1) continuous 24C; 2) continuous 4C; and 3) application of thiourea (TU)/gibberellic acid (GA) at various concentrations to seed held at 24C then subsequently chilled at 4C. Treatments of continuous 24 or 4C indicated that catalase, G6PDH, and 6PGDH exhibited significant activity increases only when the seeds obtained germination potential, which occurred in the seeds chilled for 7 weeks at 4C. Seeds held at 24C did not germinate and showed little change with time in G6PDH and 6PGDH activity. There was only a slight increase in catalase activity beginning 3 weeks following treatment initiation and a decrease in activity following 13 weeks of treatment. Thiourea treatment resulted in an inhibition of catalase activity and a stimulation of G6PDH, but had no effect on 6PGDH activity. However, no correlation between enzymic activity and seed germination was found. The results strongly questioned the role of the ppp and catalase activity in dormancy control as previously hypothesized.

It has been proposed that ppp activity plays a central role in the breaking of seed dormancy (Roberts and Smith, 1977). Direct enzymic activity measurement has shown that ppp activity increased during dormancy release (Gosling and Ross, 1980; Kovacs and Simpson, 1976; Swamy and Sandhyarani, 1986). Dormancy-breaking chemicals, such as nitrite, azide, hydroxylamine, and thiourea (TU), are assumed to increase activity of the ppp by inhibiting catalase, resulting in increased levels of H_2O_2 , which is required for the reoxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), the essential coenzyme of the ppp (Hendricks and Taylorson, 1974, 1975). Efficient bud dormancy-breaking agents (e.g., TU) decrease catalase (EC 1.11.1.6) activity in grapevine, but less efficient dormancy-breaking chemicals do not (Nir et al., 1986). Nir et al. (1986) have shown that chilling decreased catalase activity and that the rate of bud forcing at 23C was negatively related to the activity of catalase. These data suggest that the ppp plays a positive role while catalase exhibits an inhibiting role in dormancy release. However, there are data that show that the ppp is not involved in dormancy release (Adkins and Ross, 1981; Satoh and Esashi, 1980; Upadhyaya et al., 1981). Data are conflicting on the role of the pathway in dormancy release. Therefore, the present study was initiated to determine: 1) if chilling enhances ppp enzymic activity and inhibits catalase; and 2) if TU treatment, which inhibits catalase and breaks dormancy in some species (Hendricks and Taylorson, 1975; Nir et al., 1986) stimulates (at least does not inhibit) ppp enzymes in nectarine seeds.

Materials and Methods

Seed treatment. Seeds of 'Nectarine 7' nectarine were collected early in Aug. 1986, air-dried, and stored in a laboratory ($\approx 24C$) for 4 months. At the end of this period, the seeds were

subjected to the following imbibition treatments: 1) room temperature control (continuous 24C); 2) continuous chilling at $4.0 \pm 0.5C$; and 3) applications of 12 μM GA [because Erez et al. (1971) found that it may have a synergistic effect with TU in breaking dormancy] in combination with TU at either 0 (also omit GA), 1.3, 13, or 130 mM; these seeds were initially kept at $\approx 24C$, then chilled at $4 \pm 0.5C$. The first TU/GA treatment was applied (for 24 hr) on day 12, following initiation of the study; the second treatment was applied on day 23 for 48 hr; the third treatment was applied on day 55 for 12 hr. The concentration of TU for each specific treatment was the same in all three applications. All TU/GA treatments were applied by soaking the seeds in aerated TU/GA solutions for the prescribed time. The seeds of all treatments were kept in moist peat moss in flats, and each treatment was run twice.

Enzyme extraction and assay. Enzyme extraction and activity assays were done at weekly intervals. The extraction procedure was modified from Gosling and Ross (1979) and Upadhyaya et al. (1981), and the following procedure was used for all enzymes: Random samples of 10 seeds each, with endocarp removed, were weighed and ground immediately with a mortar and pestle in 19 ml extraction medium plus 1 g insoluble polyvinyl polypyrrolidone. Low temperature was maintained with an ice bath. The extraction medium consisted of 25 mM potassium phosphate buffer (Kpi buffer), pH 7.4, containing 1 mM ethylenediamine tetraacetic acid (EDTA) and 100 mM 2-mercaptoethanol. The resulting macerate was centrifuged at $12,100 \times g$ for 10 min. The supernatant was collected and brought to 20% saturation with $(NH_4)_2SO_4$ and centrifuged at $12,100 \times g$ again for 10 min. The precipitate was discarded. [Previous studies indicated that no enzymic activity was detected for the precipitate either below 20% or above 80% $(NH_4)_2SO_4$.] The $(NH_4)_2SO_4$ concentration was increased to 80% saturation, and the precipitate was collected by centrifugation at $12,100 \times g$ for 10 min and suspended in 2.5 ml 25 mM Kpi buffer, pH 7.4, then dialyzed against this buffer until free of $(NH_4)_2SO_4$. All procedures were carried out between 0 and 4C.

Received for publication 11 Jan. 1990. A contribution of the Georgia Agricultural Experiment Station, College Station, Athens. This research was supported by state and Hatch funds awarded to the Georgia Agricultural Experiment Station, College Station, Athens. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

Abbreviations: NADP, nicotinamide adenine dinucleotide phosphate; ppp, pentose phosphate pathway; TU, thiourea.

Pentose phosphate pathway enzymes were estimated by the method of Muto and Uritani (1970) with slight modification. Activities of G6PDH (EC 1.1.1.49) and 6PGDH (EC 1.1.1.44) were determined by the initial rate of reduction of NADP⁺ at 24 ± 0.5°C. The increase in absorbance at 340 nm was recorded for the initial 5 min (the reaction was linear for the first 10 rein) on a Beckman Acts CV spectrophotometer (Beckman, Fullerton, Calif.), reading sample solutions against a blank composed of buffer, NADP⁺, and enzyme extract. Two assays were carried out. One contained, in a final volume of 3.0 ml, 50 mM Tris-HCl buffer (pH 7.8), 1.7 mM glucose-6-phosphate (G6P), 1.7 mM 6-phosphogluconate (6PG), 0.2 mM NADP⁺, and 0.1 ml enzyme extract. The other contained all these ingredients except G6P. The difference in the NADP⁺ reduction rate between these two assays was used to calculate G6PDH activity. Each assay was run twice.

Catalase activity was assayed according to Worthington (1977). The disappearance of H₂O₂ was followed spectrophotometrically at 240 nm for the initial 45 and 60 sec, respectively. The final volume of the reaction mixture was 3.0 ml and consisted of 50 mM Kpi buffer, pH 7.0, 19.7 mM H₂O₂, and 0.1 ml enzyme extract. Each assay was run 3 times.

Protein was determined by biuret method (Zamenhof, 1957); bovine serum albumin was used as the standard.

Seed germination test. Seed germination tests were done at room temperature (24°C) at weekly intervals during imbibition treatments. Germination was defined as radicle emergence. After germination, the seeds were grown in vermiculite (No. 3)-filled 400-ml plastic pots. Statistical treatment of the data consisted of the calculations of standard deviations for each mean.

Results

Germination and its relationship to G6PDH, 6PGDH and catalase activity. Control seeds (unchilled seeds) did not germinate (Fig. 1). Chilled seeds began germination at 7 weeks of chilling, and germination increased progressively until about the 11th week, after which there was no increase (Fig. 2).

The G6PDH activity in control seeds (Fig. 1) fluctuated slightly during the initial 2 weeks, then remained relatively constant for the next 4 weeks. Thereafter (7th week on), the activity slowly decreased. In chilled seeds (Fig. 2) there was little difference in G6PDH activity from the control for the initial 6 weeks except week one. However, from the 7th week on, activity began to increase, and this increase in activity coincided with increased seed germination.

Fig. 1. Time course of nectarine seed germination (A) and changes in G6PDH (●; nmoles NADP⁺ reduced/rein per milligram protein), 6PGDH (□; 1 nmoles NADP⁺ reduced/rein per milligram protein) and catalase (○; micromoles H₂O₂ breakdown/rein per milligram protein) activities in the seeds of the control held at room temperature.

Fig. 2. Time course of nectarine seed germination (A) and changes in G6PDH (●; nmoles NADP⁺ reduced/rein per milligram protein), 6PGDH (□; nmoles NADP⁺ reduced/rein per milligram protein) and catalase (○; micromoles H₂O₂ breakdown/rein per milligram protein) activities in seeds held under continuous chilling at 4°C.

Fig. 3. Effects of TU/GA treatment and subsequent chilling at 4°C on nectarine seed germination. Final TU/GA treatment was applied at the 8th week. Chilling began at the 12th week (arrow). 0 mM TU/0 μM GA (A), 1.3 mM TU/12 μM GA (○), 13 mM TU/12 μM GA (●), and 130 mM TU/12 μM GA (□).

The 6PGDH in the control seeds (Fig. 1) maintained relatively constant activity during the initial 5-week treatment, but beginning in the 6th week activity slowly decreased. 6PGDH activity in chilled seeds remained basically unchanged until the 8th week, after which it exhibited a gradual increase. As with G6PDH activity, the increase in 6PGDH activity also seemed to be related to seed germination (Fig. 2).

Catalase activity in control seeds (Fig. 1) was initially very low but increased and was relatively high from the 5th to the 16th week, after which activity decreased. In the chilled seeds (Fig. 2), catalase had lower activity level than that of the control until the 7th week, after which activity increased rapidly until the 15th week. As was the case with G6PDH and 6PGDH, this rapid increase in catalase activity occurred simultaneously with seed germination.

Germination of TU/GA-treated seeds. Chilling was required for germination of TU/GA-treated seeds (Fig. 3). Two weeks of chilling (week 12 to 14) resulted in > 50% germination in seeds treated with 13 or 130 mM TU/12 μM GA, but there was no germination with the 1.3 mM TU/12 μM GA or the 0 TU/GA. After 3 weeks of chilling (week 12 to 15), some of the 1.3 mM TU/12 μM GA-treated seeds began to germinate, while seeds in the 0 TU/GA began germinating after 5 weeks of chilling.

Following 3 weeks of chilling (week 12 to 15), seeds treated with 13 mM TU/12 μ M GA germinated very rapidly and uniformly, and most seedlings in 1.3 and 13 mM TU/12 μ M GA treatments developed normally. Although total germination percentage of seeds treated with 130 mM TU/12 μ M GA was high, the seeds germinated very slowly, and all finally died (apparently due to TU toxicity).

Catalase activity in TU/GA-treated seeds. Catalase activity was initially greatly inhibited by TU/GA treatment relative to the control (Fig. 4). The inhibition was greater at the higher TU concentrations. Two weeks (week 10) after the final treatment, catalase activity was lowest for the 130 mM TU/12 μ M GA treatment and highest for the 0 TU/GA. Four weeks following the final treatment (week 12), activity increased in all three treatments but activity in the 1.3 mM TU/12 μ M GA treatment increased the most. Following 3 weeks of chilling (week 12 to 15), activity in all treatments decreased. At this point, there were no differences among treatments except for the 130 mM TU/12 μ M GA treatment. Also, following 4 weeks of chilling, different responses in activity were detected.

G6PDH activity in TU/GA-treated seeds. Unlike catalase activity, that of G6PDH was stimulated by TU/GA treatment (Fig. 5). Enhancement increased with TU concentration. After 2 weeks

following the final treatment (week 10), the greatest activity was shown in the 130 mM TU/12 μ M GA treatment (activity was even higher after the final treatment for 4 weeks), and the lowest activity level occurred in the 0 TU/GA. Chilling for 3 weeks (week 12 to 15) reduced G6PDH activity for the 130 and 13 mM TU/12 μ M GA treatments, while low activity was still maintained in the 1.3 mM TU/12 μ M GA treatment and 0 TU/GA. Following 4 and 5 weeks of chilling, G6PDH activity responded with a slight difference among the treatments, but activity remained highest in the 130 mM TU/12 μ M GA and lowest in the 0 TU/GA.

6PGDH activity in TU/GA-treated seeds. In contrast to either catalase or G6PDH activity, TU/GA treatment had no significant effect on 6PGDH activity (Fig. 6) either before or after chilling.

Discussion

It has been proposed that the release of seed dormancy is associated with an increased ppp activity (Roberts and Smith, 1977). Increased ppp participation during dormancy release in *Prunus cerasus* was shown by the C_0/C_t technique (LaCroix and Jaswal, 1967). Also, Simmonds and Simpson (1971) reported increased ppp activity in response to after-ripening and GA₃ treatment in caryopses of *Avena fatua*. A significant increase in ppp enzymes during stratification coinciding with dormancy breakage before germination in *Corylus avellana* seeds was also reported (Gosling and Ross, 1980). Similarly, Swamy and Sandhyarani (1986) demonstrated that a significant increase in the levels of the two key ppp enzymes (G6PDH and 6PGDH) during after-ripening corresponded to a concomitant increase in peanut germination. However, the correlation between dormancy release and the inhibition of catalase induced by thiourea was thought to act through the stimulation of the ppp (Hendricks and Taylorson, 1975; Nir et al., 1986). But there is no convincing evidence for this contention.

Our data demonstrate that 1) significant activity increases in G6PDH and 6PGDH, the two key enzymes in the ppp, occurred at the same time with, but not before, the increases in seed germination of chilled seeds, and 2) germination reached a maximum before maximum activity levels of the two enzymes were reached (Fig. 2). Therefore, the increased ppp activity is probably the result of dormancy breakage (a germination phenomenon) but not the cause of dormancy. This conclusion is

Fig. 4. Effects of TU/GA treatment and subsequent chilling at 4C on catalase activity of nectarine seed. Final TU/GA treatment was applied at the 8th week. Chilling began at the 12th week (arrow). O mM TU/0 μ M GA (A), 1.3 mM TU/12 μ M GA (o), 13 mM TU/12 μ M GA (●), and 130 mM TU/12 μ M GA (□). Note the scale when comparing the results with those in Figs. 1 and 2.

Fig. 5. Effects of TU/GA treatment and subsequent chilling at 4C on G6PDH activity of nectarine seed. Final TU/GA treatment was applied at the 8th week. Chilling began at the 12th week (arrow). O mM TU/0 μ M GA (A), 1.3 mM TU/12 μ M GA (o), 13 mM TU/12 μ M GA (●), and 130 mM TU/12 μ M GA (□).

Fig. 6. Effects of TU/GA treatment and subsequent chilling at 4C on 6PGDH activity of nectarine seed. Final TU/GA treatment was applied at the 8th week. Chilling began at the 12th week (arrow). O mM TU/0 μ M GA (A), 1.3 mM TU/12 μ M GA (o), 13 mM TU/12 μ M GA (●), and 130 mM TU/12 μ M GA (□).

strengthened by the fact that catalase, which was proposed to inhibit ppp (Hendricks and Taylorson, 1975; Nir et al., 1986), also showed a rapid increase in activity when G6PDH, 6PGDH activities, and germination began to increase.

When the enzymic activities were tested after the final TU/GA treatment but before chilling, the results were promising. There was a concentration response with G6PDH activity and TU treatment. Activity was promoted at higher treatment levels. Catalase also showed a concentration response but in an inverse manner, with decreased activity at higher TU levels. However, although enzymic activities responded exactly as hypothesized (Hendricks and Taylorson, 1975; Nir et al., 1986; Roberts and "Smith, 1977), the seeds still did not germinate unless they were given a chilling treatment. Also, after chilling for 3 or 4 weeks, catalase activity decreased, but G6PDH activity also decreased. Seeds germinated at low levels of G6PDH, 6PGDH, and catalase activities (Figs 3 through 6), the opposite result of the continuous chilling treatment (Fig. 2) and of previous reports (Gosling and Ross, 1980; Kovacs and Simpson, 1976; Swamy and Sandhyarani, 1986; Upadhyaya et al. 1981). This finding is important because it is the first report to demonstrate that reduced ppp activity still leads to seed germination. If the measured enzymic activity changes do indeed reflect the in vivo situation, it is evident that the release of dormancy in nectarine seed has little to do with changes in the ppp activity. This conclusion is supported by other work (Adkins and Ross, 1981; Satoh and Esashi, 1980; Upadhyaya et al., 1981; Thévenot et al., 1989). The proposed key role of catalase in dormancy control also seems unlikely in nectarine seeds. This finding is in agreement with previous ones that did not show a correlation between catalase activity and dormancy release with various approaches in cocklebur seeds (Esashi et al., 1979) and rice grains (Kuo, 1981; Kuo and Chu, 1983).

Increased ppp carbon flow has not been confirmed in recent experiments. On the contrary, Jones and Hall (1981) and Fuerst et al. (1983) reported that dormancy breaking was accompanied by- an increased C_2/C_1 ratio, indicating decreased ppp relative to glycolysis in both *Spergula arvensis* and wild oat (*Avena fatua*) seeds. Adkins and Ross (1981) attributed the increased activity of ppp shown by Gosling and Ross (1980) to be a plant response to chilling. Also, Adkins and Ross (1981) and Bewley and Black (1982) suggested that the increased activities reported by Kovacs and Simpson (1976) in nondormant wild oat grains are more likely associated with the germination process. It seems the relationship between ppp activity and dormancy release shown by Swamy and Sandhyarani (1986) can also be interpreted in the same way. Although Hendricks and Taylorson (1975) suggested a role for catalase in dormancy control, Bewley and Black (1982) pointed out several weaknesses of this theory and suggested that TU could be affecting catalase activity associated with growth rather than with germination. In addition, the actual levels of hydrogen peroxide accumulating (due to catalase inhibition) in the seed tissue during treatment were not reported in the study (Hendricks and Taylorson, 1975).

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