# Tannic Acid Effects on *Raphanus raphanistrum* Root Acid Phosphatase[1]

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**ABSTRACT. The effects of tannic acid (TA) on acid phosphatase (APase) (E.C.3.1.3.2) from root extracts of fertilized or unfertilized *Raphanus raphanistrum* plants were examined. TA inhibited APase activity non-competitively; $V_{max}$ was reduced, but the apparent $K_M$ was unaffected in the presence of added TA. Endogenous tannin content ranged between 0.26 and 0.56 μmole·g fresh weight$^{-1}$ of root tissue. Enzyme activity data was extrapolated to provide estimates of activity in the absence of any TA; uncorrected APase activity of *R. raphanistrum* was underestimated by an average of 25%. Addition of polyvinylpyrrolidone (PVP) did not affect measured enzyme rates in a consistent manner.**

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## INTRODUCTION

Tannins and other phenolic compounds present in crude extracts prepared from plant tissues may interfere with measurement of enzyme activity. Rao and others (1996, 1998) reported that TA inhibited the *in vitro* activity of purified sweet potato APase.

Hall and others (1998) previously reported that APase activity measured in crude extracts of *R. raphanistrum* roots was elevated in plants harvested from unfertilized successional field plots. Elevated APase activity has been observed in many other plant species growing under phosphorus-limiting conditions (Duff and others 1994).

However, if tannin levels in plants extracts used in these studies were high enough to affect estimates of APase activity, then enzyme activity might be underestimated. The objectives of this study were to measure the TA levels in root extracts prepared from field grown *R. raphanistrum*, to determine if the TA levels were high enough to interfere with enzyme activity, and to derive a method for transforming measured APase enzyme data to more accurately reflect endogenous APase activity.

## MATERIALS AND METHODS

Thirty experimental plots (20 × 16 m) were established in a field previously under cultivation. Control (C) plots received no nutrient enrichment. Other experimental plots were treated with either nitrogen or nitrogen-phosphorus (NP) fertilizer (300 kg·ha$^{-1}$ nitrogen as $NH_4NO_3$ and 768 kg·ha$^{-1}$ phosphorus as $(NH_4)_2HPO_4$). The timing of fertilizer application was either annual or on a one-time basis (year 1, 3, or 5).

Fifty or more *R. raphanistrum* plants were harvested from each of three C plots and three NP plots on three dates: one week prior to, one week after, and two weeks after fertilizer treatment (21 May) during the 1998 season. Excess soil was removed from roots by rinsing in deionized water. Fresh weights of roots and shoots were measured. Roots were ground in cold 50 mM sodium acetate (NaOAc) buffer, pH 5.7, using either 5.0 ml or 10.0 ml buffer per g of root tissue. Extracts were clarified by centrifugation at 10,000 *g* at 4° for 10 min. Supernatants were collected, and pellets recentrifuged under the same conditions. Supernatants were combined and the extract stored at -80° until use. Root mass concentration was calculated as g per ml final volume of extract.

In some experiments PVP was added to extracts to a concentration of 2% w/v. After it dissolved, residual precipitate was removed by centrifugation.

The Price-Butler method for tannin analysis as reported by Waterman and Mole (1994) was slightly modified for this study. Sample extract (0.1 ml) was added to 5.0 ml $H_2O$, then 0.6 ml 0.1 M $FeCl_3$ (in 0.01 M HCl) and 0.6 ml 8 mM $K_3Fe(CN)_6$ were added in succession. Absorbance at 720 nm was measured after 15 min. The molar TA equivalent was calculated from a standard curve, correcting for the NaOAc buffer included in the plant extract.

Previous studies of this method (K. Yu and C. Fried, personal communication) indicated that the originally recommended 3 min delay between the addition of $FeCl_3$ and $K_3Fe(CN)_6$ reagents was unnecessary, provided that tubes were inverted after the addition of the last reagent. Also, assay temperature slightly affected results; hence, assays were run at the same ambient temperature.

APase activity was monitored as the formation of *para*-nitrophenol (*p*-NP) from the substrate *para*-nitrophenyl phosphate (*p*-NPP). Reaction mixtures contained 12.5, 25, or 50 μl of crude root extract in 50 mM NaOAc buffer, pH 5.7, and 10 mM $MgCl_2$, plus varying amounts of TA (0-10 μM). *p*-NPP (15 mM) was added to initiate the reaction. Total reaction volume was 0.5 ml. Reaction mixtures were incubated at 37° for 5.0 min, and were terminated by the addition of 2.0 mL 0.5 M NaOH. Product formation was followed at 405 nm ($\lambda_{max}$ nm (log ε): 405 (4.27)) and enzyme activity was measured as μmoles of *p*-NP·sec$^{-1}$·g protein$^{-1}$. Commercial grade TA (0-20 μM) was used for inhibition studies. For kinetic studies, pooled extracts from C- and from NP-treated plants harvested 1 week post-treatment were used. Reaction mixtures for kinetic studies included 0-20 mM *p*-NPP and 0 or 10 μM added TA.

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Protein was determined using BSA as the standard in the Bradford procedure (Bradford 1976). Absorbance was linear between 1.25 and 12.5 μg/ml of BSA.

## RESULTS

The rate of *p*-NP formation by APase in crude extracts prepared from roots of *R. raphanistrum* increased to a maximum velocity of 38-40 μkat·g protein$^{-1}$ as substrate concentration was increased to 20 mM (Fig. 1). Added TA (10 μM) inhibited APase activity at all substrate levels (2.0 to 20 mM). A velocity of 20 μkat·g protein$^{-1}$ was reached at 20 mM substrate and 10 μM TA. Inhibition of *R. raphanistrum* APase by added TA was independent of fertilizer treatment or plant age (data not shown).

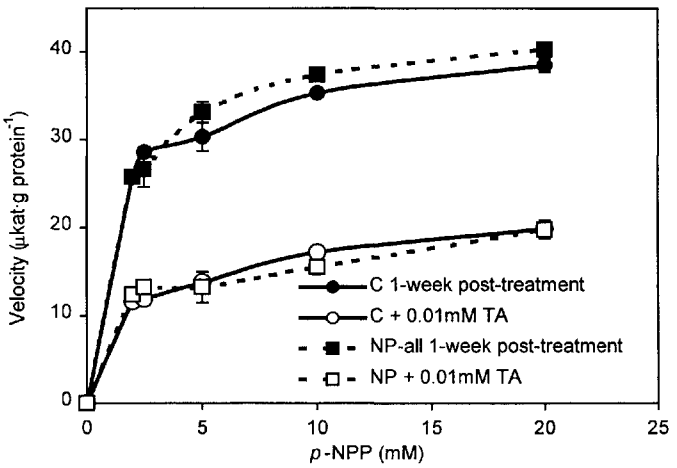


FIGURE 1. Rates of *p*-NP formation in the presence or absence of 10 μM TA, in response to increased concentration of *p*-NPP. Extracts used in this experiment were pooled samples, representing plants harvested from three different experimental plots for each treatment (C or NP) one week after fertilizer application. Each point represents the mean ± s.e. of three determinations.

The TA content of root extracts was measured using a modified Price-Butler procedure (Table 1). TA equivalents present in root extracts ranged from 0.37 to 0.56 μmol·g fresh weight$^{-1}$. TA content did not change significantly as a function of treatment or plant age. Therefore, in a typical enzyme assay TA content was approximately 5 μM TA equivalents.

Inhibition of APase activity was measured at saturating substrate concentration (15 mM) in the presence of increasing TA concentration. In Figure 2, results averaged for all samples irrespective of age are shown. Residual APase activity, defined as the ratio of activity observed in the presence of added TA compared to that in the absence of added TA (designated as endogenous activity), was progressively inhibited by added TA in all samples. Residual activity in C samples reached the 50% level between 15 and 20 μM TA while NP-treated samples retained more than 50% residual activity at the same concentrations of TA.

Some extracts were prepared in buffer containing PVP, a tannin-binding compound. The effects on TA levels and APase activity of added PVP were measured. In Table 1 it can be seen the addition of PVP to extracts reduced measured levels of TA eq in each sample, however the degree of reduction was variable. In Figure 3A the solid circles (●) show that APase activity in C samples was enhanced from 7 to 23% when PVP was added to the extraction buffer. The effect on NP samples was more variable, even reducing measured activity in some (Fig. 3B).

TABLE 1

*Tannin content of* R. raphanistrum *root extracts.**

| Treatment | Collection Time | -PVP | +PVP |
|---|---|---|---|
| Control | Pre-fertilizer | 0.44 ± 0.05 | 0.35 ± 0.03 |
| | Post-fertilizer 1 week | 0.43 ± 0.12 | 0.40 ± 0.11 |
| | Post-fertilizer 2 weeks | 0.56 ± 0.04 | 0.48 ± 0.04 |
| NP | Pre-fertilizer | 0.42 ± 0.06 | 0.33 ± 0.04 |
| | Post-fertilizer 1 week | 0.37 ± 0.03 | 0.28 ± 0.04 |
| | Post-fertilizer 2 weeks | 0.40 ± 0.07 | 0.34 ± 0.03 |

*Root tannins were extracted in the presence or absence of PVP and measured as described in Materials and Methods. Values are given as mean ± s.e. TA equivalents (μmoles·g fresh weight of root$^{-1}$).

## DISCUSSION

Plant growth in old-field successional plots was studied by McClure and Benninger-Truax (1996) in

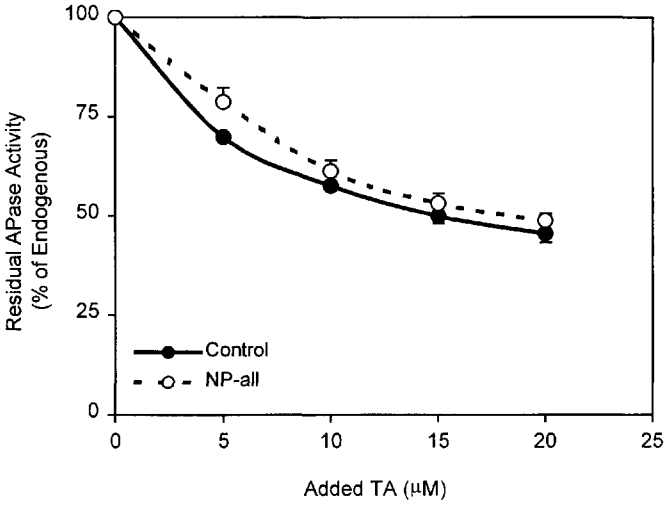


FIGURE 2. Effect of added TA on APase activity. The data represent mean ± s.e. APase activity observed for all samples irrespective of age. Activity is expressed as the ratio of activity remaining in the presence of TA relative to endogenous activity.

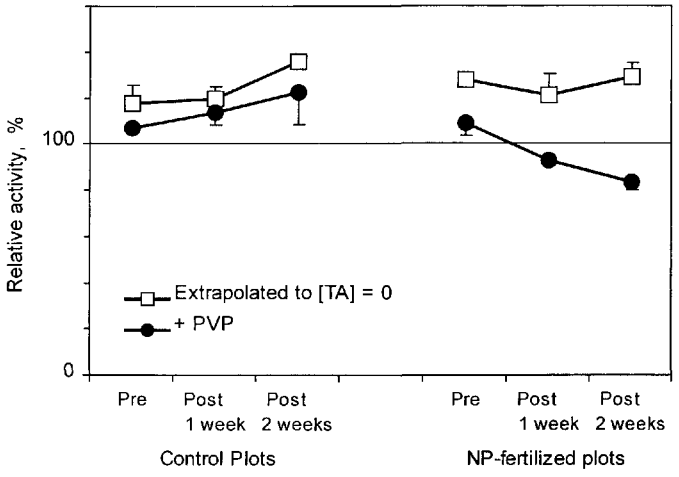


FIGURE 3. APase activity from C and NP extrapolated to TA = 0 (□) compared to APase activity in extracts prepared with PVP (●). Data points are shown relative to endogenous activity which is taken as 100%. Each point represents the mean ± s.e. of three determinations from each of three plots.

TABLE 2

*Kinetic constants.**

| Sample | $V_{max}$ (μkat·g protein$^{-1}$) | $K_M$ (mM) |
|---|---|---|
| C | 39.8 ± 0.5 | 1.12 ± 0.08 |
| C + TA | 20.7 ± 0.5 | 1.82 ± 0.17 |
| NP | 42.0 ± 0.2 | 1.45 ± 0.04 |
| NP + TA | 18.9 ± 0.8 | 1.34 ± 0.56 |

*Data shown in Figure 1 were used to determine $V_{max}$ and $K_M$ by four different methods (Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf, and Cornish-Bowdin plots). Values of kinetic constants are given as mean ± s.e. derived from these four independent determinations.

which the progress of succession was perturbed by N or NP fertilizer treatment. They noted that species diversity decreased in NP-treated plots while the biomass of certain weedy species, including *R. raphanistrum*, increased. Acquisition of phosphorus and nitrogen by plants growing in old fields has been of interest to plant ecologists because nutrient status affects community composition, species richness, and plant biomass (Tilman and others 1996).

Many authors have noted that plants starved for phosphorus typically exhibit elevated APase activity (Ascencio 1997; Dinkelaker and Marschner 1992). In 1997 (Year 3 of the Hiram study), Hall and others (1998) reported that root APase activity of *R. raphanistrum* after NP treatment, but prior to flowering, was decreased relative to that for C plants. The present study was undertaken to better understand APase and to examine the effects of secondary metabolites present in the plant on APase activity.

APase activity in crude extracts of *R. raphanistrum* roots exhibited typical Michaelis-Menten kinetics similar to results reported for purified sweet potato APase (Rao and others 1998). Added TA reduced the maximum velocity seen at all substrate concentrations. Michaelis constants were determined by transformation of velocity data versus substrate concentration data using four different methods (Table 2). $K_M$ values were not substantially different in the presence of 10 μM TA, whereas TA inhibited $V_{max}$ 44% in NP samples and nearly 50% in C samples. These data show that TA inhibition of *R. raphanistrum* APase activity is not competitive; the unchanged $K_M$ suggests inhibition is noncompetitive.

Tannin content of *R. raphanistrum* root extracts varied with treatment (Table 1). By two weeks after fertilizer treatment, tannin content of NP treated plants was less than that of C plants. Tannin content of C plants at two weeks was greater than before fertilizer treatment. Since APase activity also increased in C plants, the question of how C plants overcame APase inhibition by tannins was raised. The observed data might be explained by expression of APase isoenzymes that are not subject to inhibition by tannins. Multiple bands of APase activity on non-denaturing gels has been observed (K. Dillon, personal communication). The possibility that APase isoenzymes, less susceptible to TA inhibition, and expressed under different conditions and at different ages, should be investigated.

Because tannins in extracts occurred at detectable levels (Table 1) and in amounts sufficient to interfere with APase activity (Fig. 2), two further studies were undertaken to better estimate AP activity. First, PVP was added to extraction buffer as PVP is known to bind tannins in solution. The results were variable. Added PVP only reduced measured levels of TA equivalents 76 to 93% of the amount measured in the absence of PVP (Table 1). Although APase activity was enhanced between 7 and 23% in C samples, in NP samples the effect was more variable, and activity was even reduced in some samples (Fig. 3).

An alternative approach was to use data for enzyme activity measured at different TA concentrations to derive a value for activity that might be observed if no TA were present. Residual APase activity declined exponentially as TA concentration increased (Fig. 2). When activity was plotted as a function of the total TA content (endogenous TA equivalents plus exogenous TA) present in a reaction mixture, data points could be fitted to an exponential function and the catalytic rate at TA = 0 could be determined (Fig. 4). This value was interpreted as the level of enzyme activity that would be predicted in the absence of any TA and has been designated as the extrapolated activity for that sample. Average extrapolated specific activities for APase are given in Table 3. Here it is seen that C samples had a higher specific activity than NP samples prior to fertilizer treatment, but that this pattern was reversed at later sampling times. This contradicts previous data from the same plots collected during the 1997 season (Hall and others 1998), and does not support our hypothesis about the role of root APase under phosphate-limiting conditions.

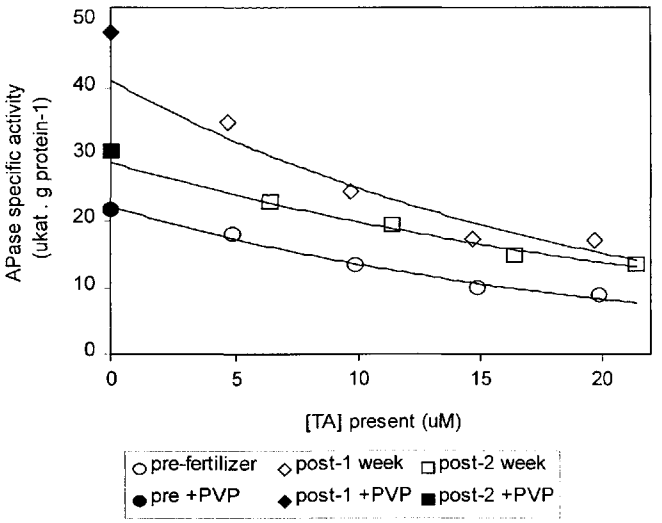


FIGURE 4. Comparison of PVP treatment with extrapolated APase activity for NP-treated plants. The open symbols, O, ◊, and, □, represent data points from samples collected at three different times, pre-fertilizer, one week post-fertilizer, and two weeks post-fertilizer treatments, respectively, but for which activity was measured in the presence of added TA. Data points are connected by the best-fit exponential curves and curves are extrapolated to TA = 0. Closed symbols, ●, ♦, and ■, give APase activities measured in the presence of PVP of samples collected at the corresponding times.

TABLE 3

*Extrapolated APase specific activity.**

| Sample | Time | | |
|---|---|---|---|
| | Pre-fertilizer | Post-fertilizer | |
| | | 1 week | 2 weeks |
| C | 33.6 ± 5.8 | 31.2 ± 4.4 | 33.8 ± 0.4 |
| NP | 24.6 ± 1.1 | 44.4 ± 3.8 | 41.2 ± 9.2 |

*Mean (± s.e.) APase activity (μkat g protein$^{-1}$) was corrected from endogenous levels by extrapolating data to TA = 0. The values represent averaged data from three plots for each treatment and time period.

A comparison of enzyme activity data (Fig. 3), either measured in the presence of added PVP or derived by extrapolation to TA = 0, emphasizes the variable PVP effect. APase activity estimated by extrapolation always exceeded that seen in PVP-treated samples, but after fertilizer treatment the measured and extrapolated data varied unpredictably. Overall, however, the trends in extrapolated data were similar for C and NP samples (Fig. 3). This suggested that extrapolation of data to the point at which TA = 0 provided a more reliable indicator of APase activity.

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