IN VIVO ASSAY OF LEAF NITRATE REDUCTASE ACTIVITY IN COCONUT *(COCOS NUCIFERA)* SEEDLINGS*

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ABSTRACT

The *in vivo* nitrate reductase assay of leaf discs of five-to twelve-month old coconut seedlings was optimized in terms of the concentrations of nitrate and dodecyl sulfate, pH, temperature, incubation time, presoaking treatment and other experimental conditions. Enzyme activity was maximal in 0.08% sodium dodecyl sulfate and 0.1M sodium nitrate at pH 8.0 and was increased by presoaking the leaf discs in 0.1M phosphate (ph 8.0), especially in the presence of 0.08% sodium dodecyl sulfate.

INTRODUCTION

Nitrate reductase (E.C. 1.6.6.1) has been assayed in many crop plants such as wheat (1,2), sugarcane (3,4), spinach (5), pea (6), corn and soybean (7, 8). However, the nitrate reductase of perennial trees has been less studied. For some fruit and forest trees, enzyme activity has been reported to be relatively low (9, 10, 11). This enzyme is known to play an important role in the utilization by plants of applied nitrogenous fertilizer. The assay of this enzyme early in the development of the plant has been proposed as an important criterion in the selection of better crop varieties, as diagnostic tool for assesing the nitrogen requirements of trees and field crops (13).

Nitrate reductase activity may be determined using either the *in vitro* or *in vivo* technique. *In vivo* assay has been reported to be the more suitable technique since it reflects better the physiological nitrate reductase activity (10, 14). Furthermore, it is more sensitive (15), less expensive and has better precision (14) than the *in vitro* technique.

The present paper deals with optimization of the *in vivo* nitrate reductase assay of leaves of coconut seedlings. The effects of the concentration of nitrate and dodecyl sulfate, pH, temperature, incubation time, presoaking and other experimental conditions on enzyme activity were studied.

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MATERIALS AND METHODS

Five-to twelve-month old coconut seedlings (Laguna variety), which have grown in clay pots or plastic bags, were used as enzyme source. The young fully open leaves were punched with a paper puncher (Lion No. 1, Japan) in order to make leaf discs (5 mm. diameter). Several leaf discs (see Results and Discussion) were transferred to a four-drams vial containing 3 ml of the assay medium. The vial was then capped, wrapped in aluminum foil to exclude light and incubated for the desired time interval.

Nitrate reductase assay was done as follows: A 0.1 ml aliquot of the supernatant in the leaf disc suspension mentioned above was mixed with 0.5 ml of 1% sulfanilamide solution (in 3N HCl) and 0.5 ml of 0.02% N-napthylethylene diamine and the mixture was vigorously shaken. The color was allowed to develop for 15 minutes after which the solution was diluted with distilled water. The absorbance was measured at 540 nm (16). nitrite reductase activity was expressed as micromoles of nitrite produced per hour per gram fresh weight of leaf discs. Assay conditions were varied in order to maximize nitrite production. As control, nitrate was omitted from the assay medium. Chloramphenicol was added to a final concentration of 10 mg/1 to the presoaking, assay and control medium. Whenever presoaking was done on the leaf discs, the latter were rinsed with 3 ml of fresh soaking medium prior to the assay.

RESULTS AND DISCUSSION

The optimal nitrate concentration for *in vivo* nitrate reductase (NR) activity of coconut leaf was found to be 0.1M in potassium phosphate buffer (0.1M, pH 7.5) at 30°C as shown in Figure 1, in agreement with the results of other workers (10, 14, 17). This nitrate concentration was used for further optimization of the enzyme assay in the present study.

Addition of sodium dodecyl sulfate (SDS) to the assay medium greatly increased NR activity and the optimal SDS concentration in the assay medium was observed to be 0.08% (Figure 2). The ratio of NR activities at 0.08 and 0% SDS is about 18. At an SDS concentrations of 0.2%, NR activity was almost nil. SDS is an anionic detergent and has been used to denature and dissociate protein subunits such as in SDS-polyacrylamide gel electrophresis and is capable of disaggregating cell walls (18). It probably changes cell membrane permeability causing faster nitrate diffusion into the cell and/or nitrite diffusion out of the cell. The effect of detergents in increasing NR activity had been demonstrated for 'Neutronyx' (14) and 'Tween' (19). The addition of sodium dodecyl sulfate to the potassium phosphate buffer (0.1M, pH 8.0) containing 0.1M KNO_3 resulted in the formation of a white precipitate. Substitution of potassium by sodium ions in the assay medium did not result in precipitation and NR activity was not altered. Succeeding assays were therefore done in sodium phosphate buffer.

Addition of from 1 to 5% n-propanol to the assay medium caused a

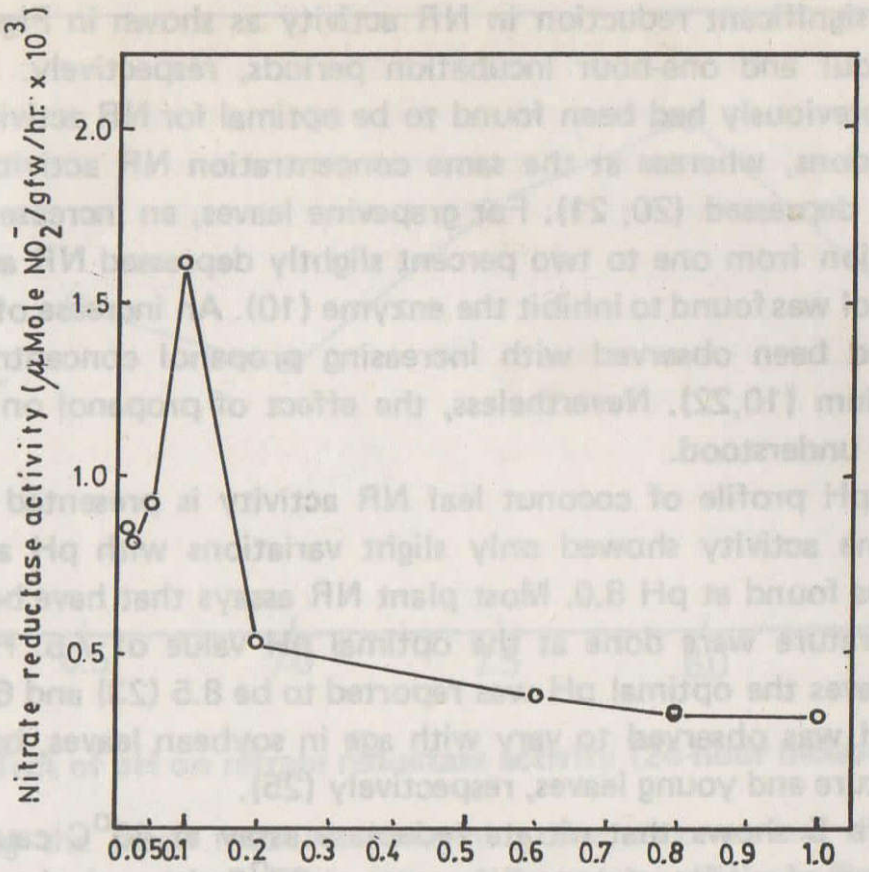

Figure 1. Effect of KNO_3 concentration (M) on nitrate reductase activity (one hour incubation).

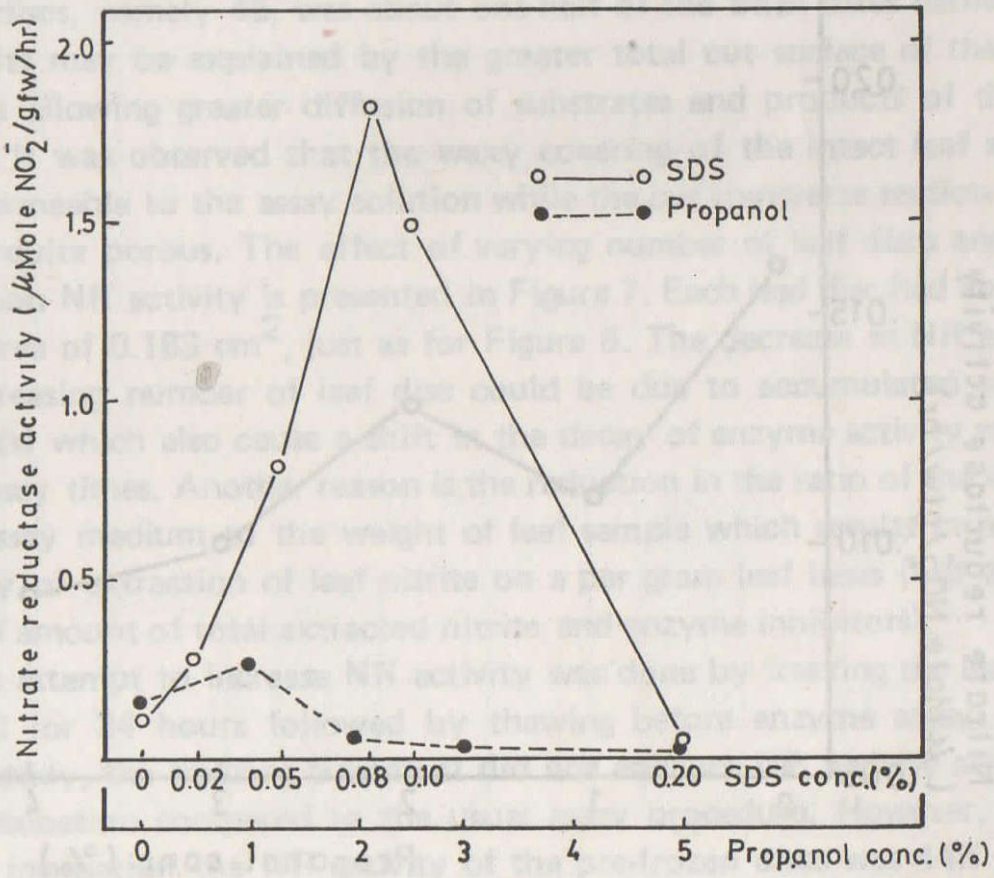

Figure 2. Effect of sodium dodecyl sulphate (SDS) and propanol on nitrate reductase activity (24-hour incubation)

small but significant reduction in NR activity as shown in Figures 2 and 3 after 24-hour and one-hour incubation periods, respectively. Five percent propanol previously had been found to be optimal for NR activity in cucumber cotyledons, whereas at the same concentration NR activity in tomato leaves was depressed (20, 21). For grapevine leaves, an increase in propanol concentration from one to two percent slightly depressed NR activity while 5% propanol was found to inhibit the enzyme (10). An increase of chlorophyll leakage had been observed with increasing propanol concentration in the assay medium (10,22). Nevertheless, the effect of propanol on NR activity is not fully understood.

The pH profile of coconut leaf NR activity is presented in Figure 4. The enzyme activity showed only slight variations with pH and maximal activity was found at pH 8.0. Most plant NR assays that have been reported in the literature were done at the optimal pH value of 7.5. However, for soybean leaves the optimal pH was reported to be 8.5 (23) and 6.5 (24). The optimal pH was observed to vary with age in soybean leaves, being 6.0 and 7.5 for mature and young leaves, respectively (25).

Figure 5 shows that nitrate reductase assay at 40°C caused a faster accumulation of nitrite compared to assay at 27°C although the decay in NR activity after 20 hours incubation was also faster at the higher temperature. The enzyme activity at 50°C was substantially less than at the lower temperature.

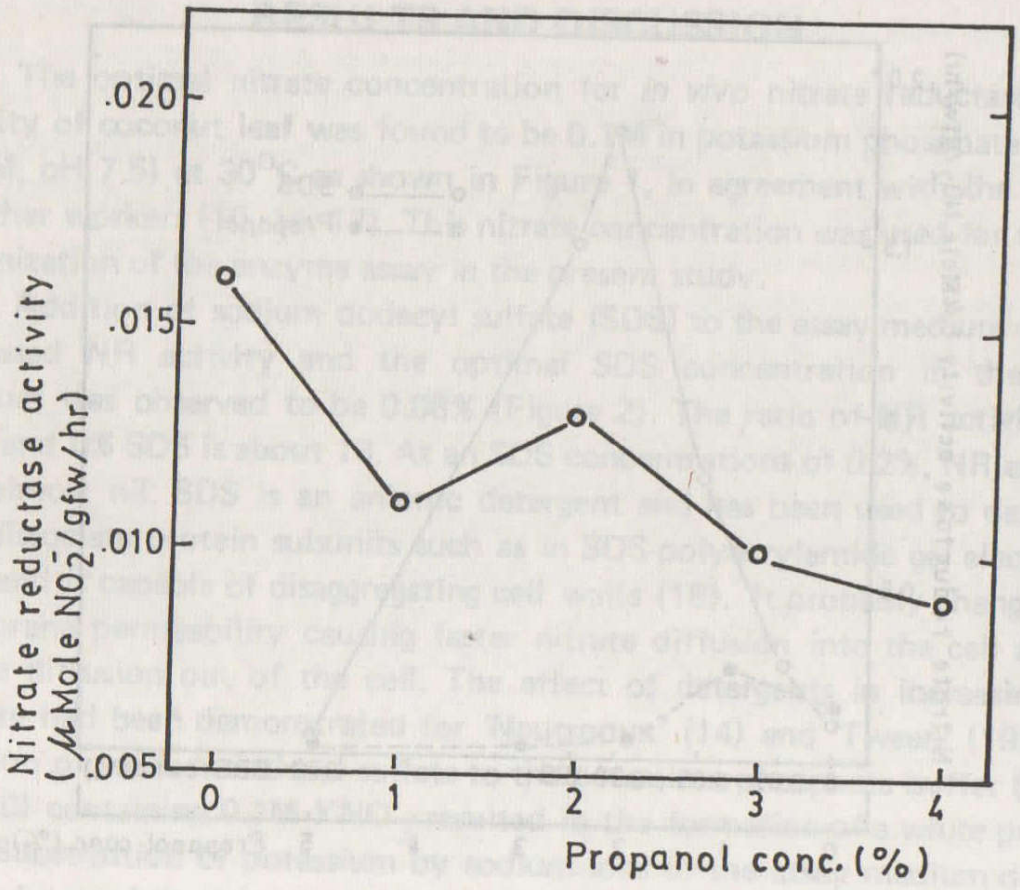

Figure 3. Effect of propanol on nitrate reductase activity (one hour incubation)

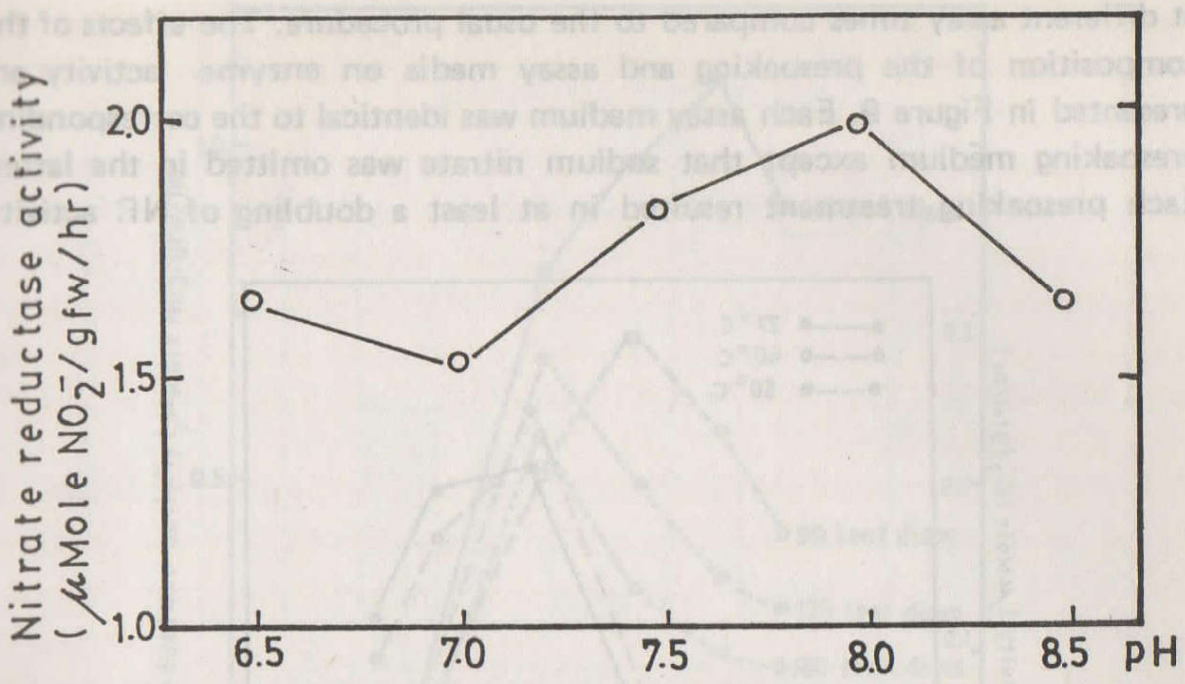

Figure 4. Effect of pH on nitrate reductase activity (24-hour incubation).

Shaking the assay medium containing the coconut leaf discs during incubation did not significantly affect NR activity (Figure 6). However, increasing the total number of leaf discs assayed with the same total leaf area (upper surface only) enhanced enzyme activity by at least 29% as shown in Figure 6. Each large leaf discs had approximately twice the upper surface area equal to 0.183 cm^2 as the small disc (0.094 cm^2) but the total number of large discs, namely 45, was about one-half of the small discs namely 88. The results may be explained by the greater total cut surface of the small leaf discs allowing greater diffusion of substrates and products of the NR reaction. It was observed that the waxy covering of the intact leaf surface was impermeable to the assay solution while the cut transverse section of the leaf was quite porous. The effect of varying number of leaf discs and total leaf area on NR activity is presented in Figure 7. Each leaf disc had an upper surface area of 0.183 cm^2, just as for Figure 6. The decrease in NR activity with increasing number of leaf disc could be due to accumulated enzyme inhibitor(s) which also cause a shift in the decay of enzyme activity towards earlier assay times. Another reason is the reduction in the ratio of the volume of the assay medium to the weight of leaf sample which results in reduced efficiency of extraction of leaf nitrite on a per gram leaf basis (but with an increased amount of total extracted nitrite and enzyme inhibitors).

An attempt to increase NR activity was done by freezing the leaf discs at -15°C for 24 hours followed by thawing before enzyme assay. In the present study, the freezing treatment did not enhance NR activity after nine hours incubation compared to the usual assay procedure. However, after a 24-hour incubation the NR activity of the pre-frozen discs was 44% greater than that using the usual procedure (Figure 8). The freezing treatment has been found successful in algae (23). It is also shown in Figure 8 that pre-soaking the leaf discs in buffer for 24 hours resulted in higher NR activities

at different assay times compared to the usual procedure. The effects of the composition of the presoaking and assay media on enzyme activity are presented in Figure 9. Each assay medium was identical to the corresponding presoaking medium except that sodium nitrate was omitted in the latter. Each presoaking treatment resulted in at least a doubling of NR activity

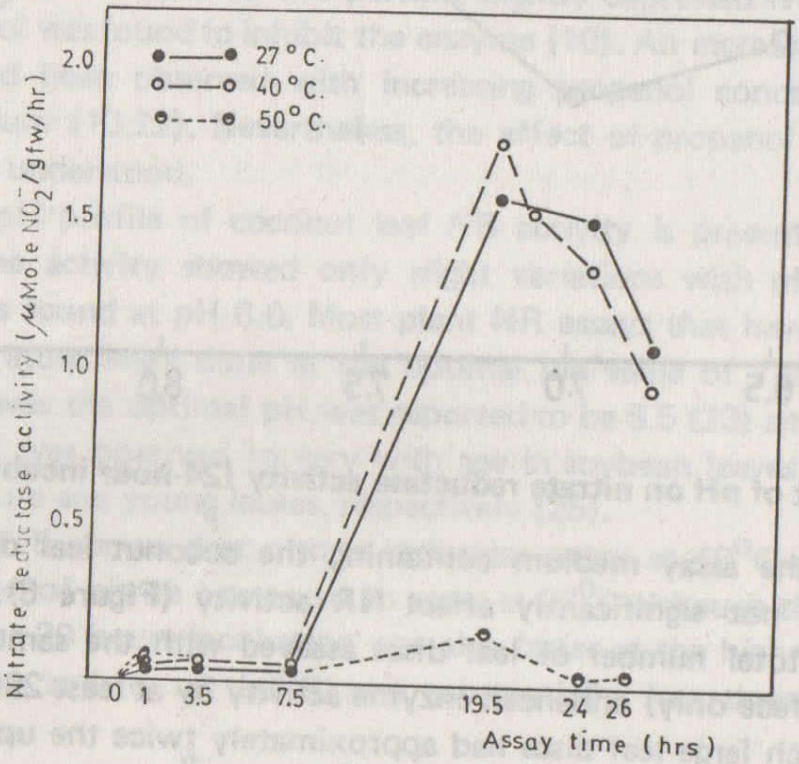

Figure 5. Effect of assay temperature on nitrate reductase activity.

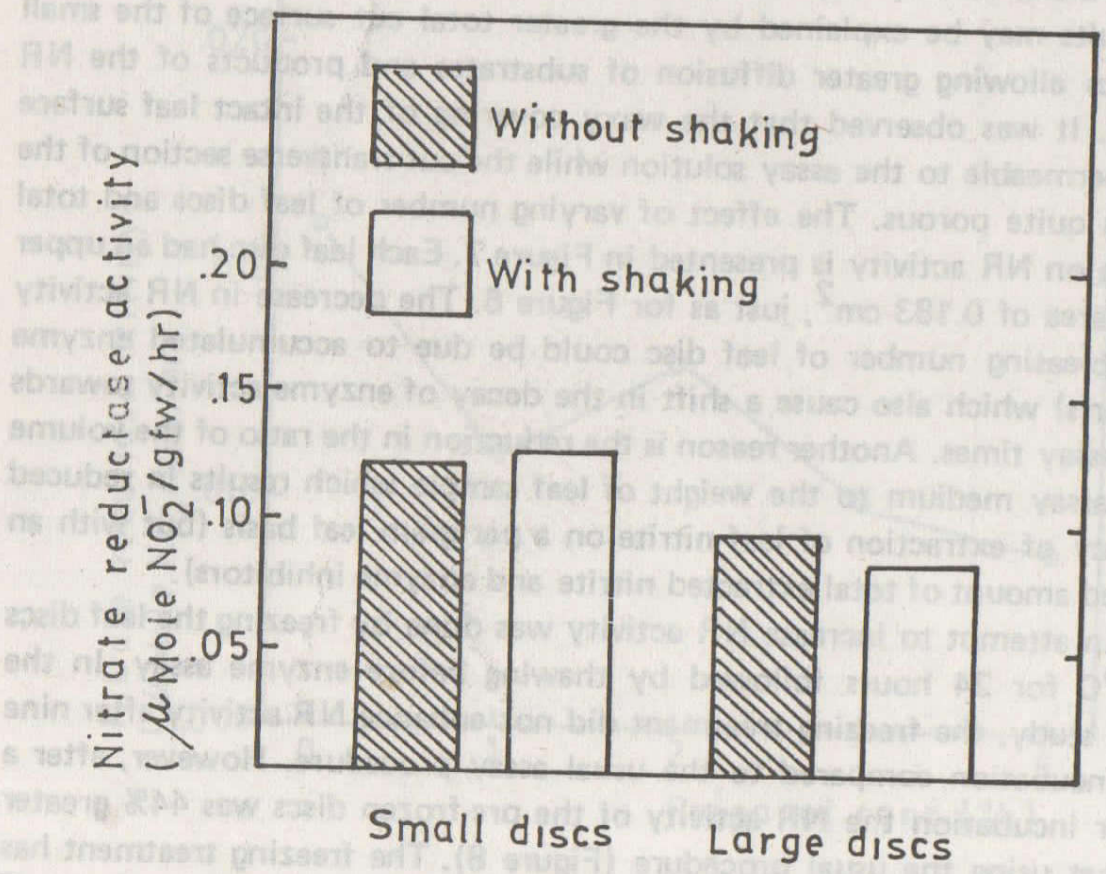

Figure 6 Effect of shaking and size of leaf discs on nitrate reductase activity (one-hour incubation).

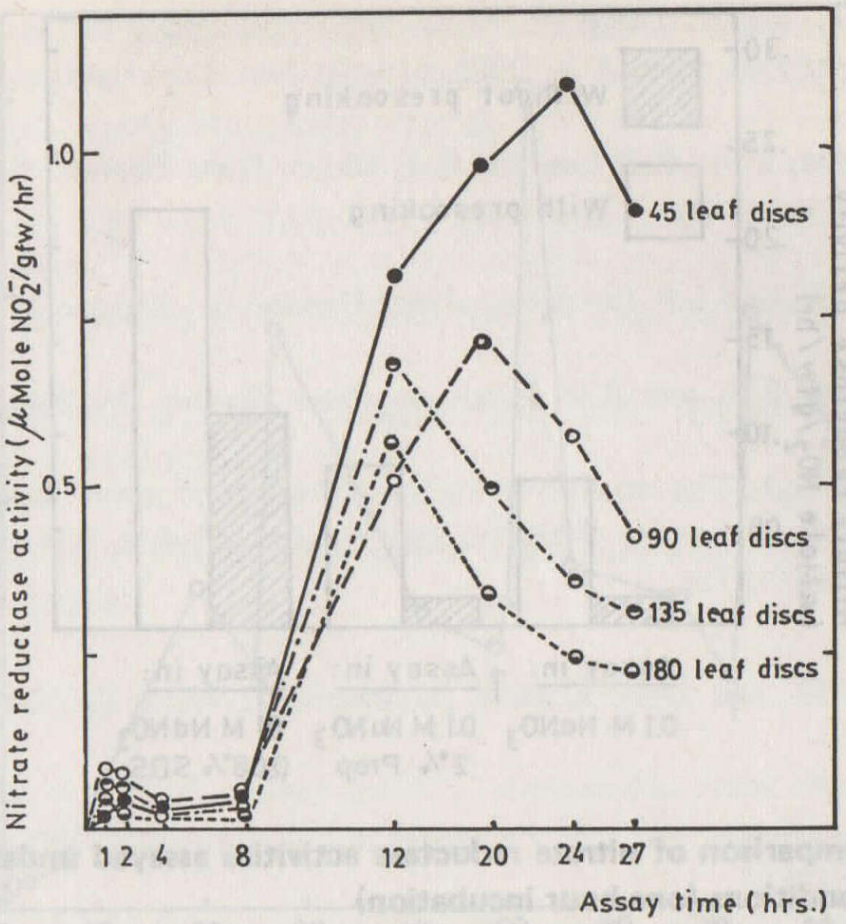

Figure 7. Effect of number of leaf discs in assay medium on nitrate reductase activity.

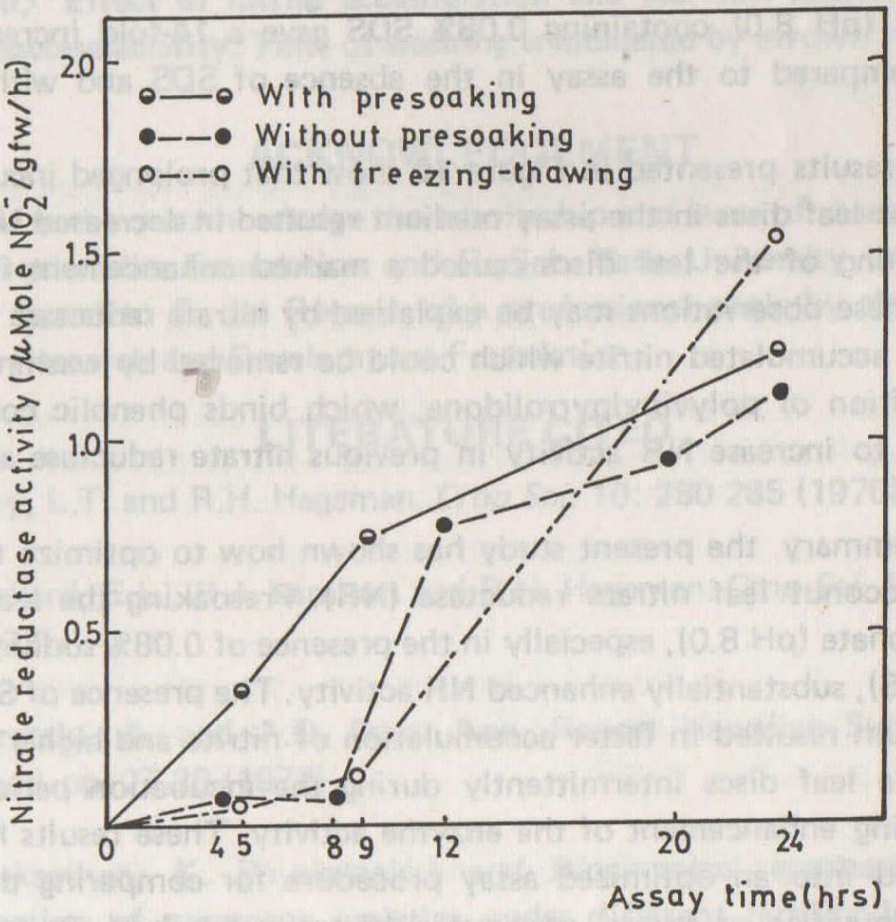

Figure 8. Effect of presoaking and freezing-thawing of leaf discs on nitrate reductase activity)

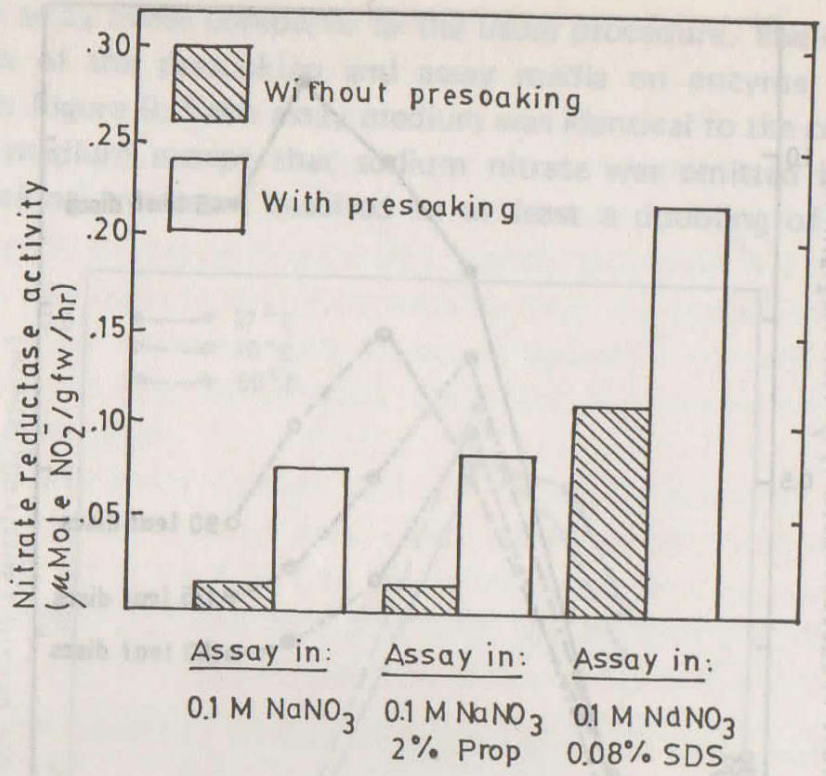

Figure 9. Comparison of nitrate reductase activities assayed under different assay conditions (one-hour incubation)

compared to the assay without presoaking. Assay in the presence of 0.08% sodium dodecyl sulfate (SDS) and 0.1M $NaNO_3$ which had been presoaked in the buffer (pH 8.0) containing 0.08% SDS gave a 14-fold increase in NR activity compared to the assay in the absence of SDS and without prior soaking.

The results presented in Figure 10 show that prolonged incubation of the coconut leaf discs in the assay medium resulted in decreased NR activity while washing of the leaf discs caused a marked enhancement in enzyme activity. These observations may be explained by nitrate reductase inhibition due to the accumulated nitrite which could be removed by washing the leaf discs. Addition of polyvinylpyrrolidone, which binds phenolic compounds, was found to increase NR activity in previous nitrate reductase assays (26, 27, 28).

In summary the present study has shown how to optimize the *in vivo* assay of coconut leaf nitrate reductase (NR). Presoaking the leaf discs in 0.1M phosphate (pH 8.0), especially in the presence of 0.08% sodium dodecyl sulfate (SDS), substantially enhanced NR activity. The presence of SDS in the assay medium resulted in faster accumulation of nitrite and higher activities. Washing the leaf discs intermittently during the incubation period caused corresponding enhancement of the enzyme activity. These results have been incorporated into an optimized assay procedure for comparing the nitrate reductase activities of several coconut cultivars and hybrids in an attempt to correlate these activities with copra yields and other agronomic characteristics. (H. Hartiko, Ph D. thesis in progress).

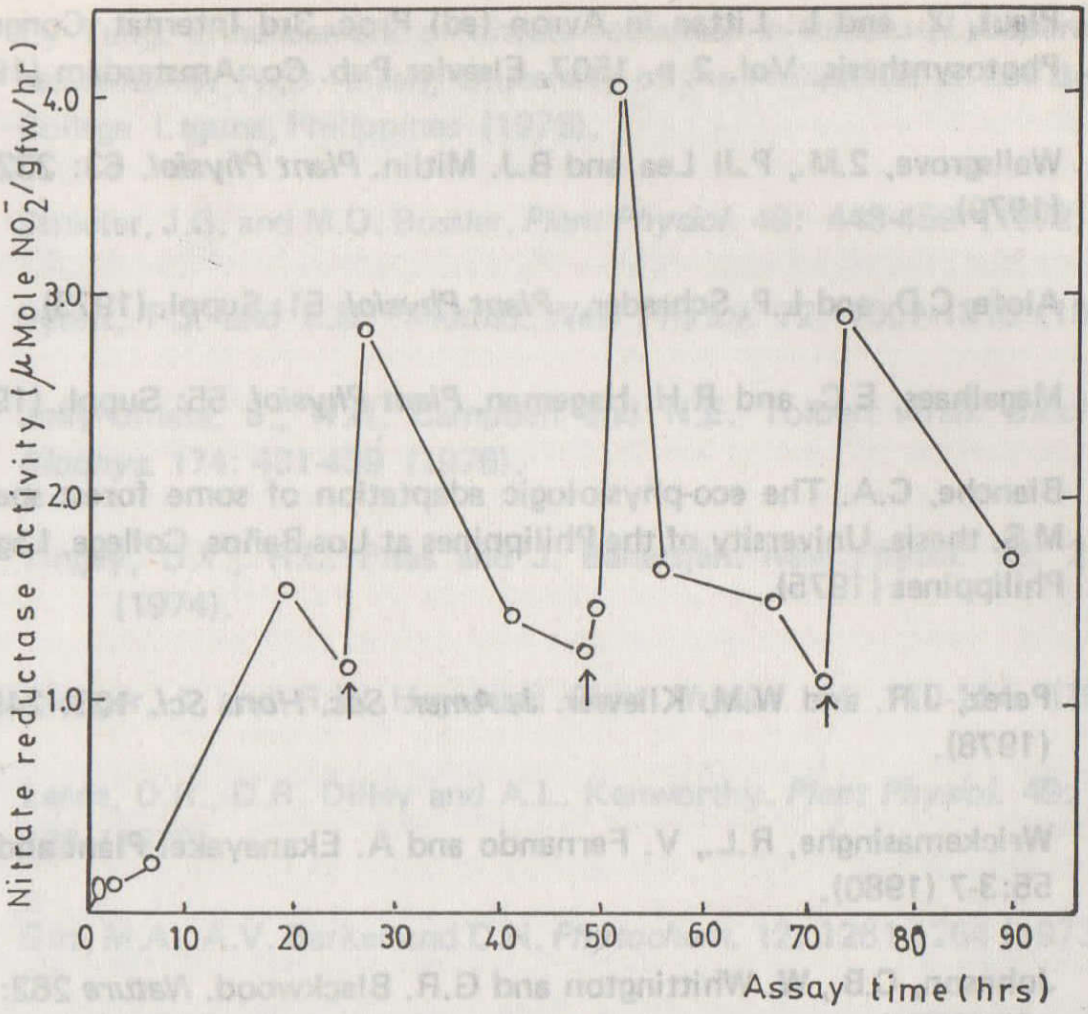

Figure 10. Effect of nitrite accumulation and leaf-disc washing on nitrate reductase activity. Time of washing is indicated by arrow.

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