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SHORT COMMUNICATION

NITRATE REDUCTASE ACTIVITY AS AN INDICATION OF MOLYBDENUM LEVEL AND REQUIREMENT OF CITRUS PLANTS*

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Abstract—Nitrate reductase activity determined in the leaf fragments of citrus plants, grown in gradually decreasing amounts of Mo in the solution culture, was used for the estimation of Mo level of the leaves. The sensitivity of the assay was tested in comparison with the chemical analysis by means of dithiol or the bioassay with *A. niger*, and was found satisfactory. The rate of increase in nitrate reductase activity in response to Mo infiltration into the leaf fragments was negatively associated with the Mo level of the substrate and the leaf tissue, and it is suggested as a quick test for the determination of Mo requirement of the corresponding trees.

INTRODUCTION

FOLLOWING the demonstration of nitrate reductase (NaRA) activity in citrus leaf fragments,[1] the application of NaRA assay as a tool for the determination of nitrogen requirement of citrus trees has been suggested.[2] Since NaRA is Mo-dependent enzyme[3,4] and its inducible nature regarding Mo has been established also for citrus leaves,[5] it was thought that the same assay might also be a valuable indicator for estimating Mo nutrition status of citrus trees. The method suggested here may be regarded also as one in a series[2,6] of attempts to elucidate the nature of "active" or "inactive" forms of a tested element in plant tissues, so that leaf analysis—as a diagnostic tool—becomes more effective.[2,6]

However, besides this fundamental problem of leaf analysis as a whole, the chemical determination of Mo in plants poses some special difficulties, e.g. limited material, risk of contamination during concentration, difficulty of manipulation,[7] especially so in citrus where its concentration in the leaves can be as low as 0·02 ppm.[8] Bolle-Jones[9] suggested that bioassay with *Aspergillus niger* of foliar Mo might be of assistance in revealing distinct depression of Mo in leaves, where chemical analysis using dithiol and amyl acetate failed to

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[2] A. BAR-AKIVA and J. STERNBAUM, *Plant Cell Physiol.* **6**, 575 (1965).
[3] D. J. D. NICHOLAS and A. NASON, *Plant Physiol.* **30**, 135 (1955).
[4] E. G. MULDER, R. BOXMA and W. L. VAN VEEN, *Plant Soil* **10**, 335 (1959).
[5] A. BAR-AKIVA, J. STERNBAUM, A. SHAKED and RUTH LAVON, *Spec. Bull.* (mimeo) 34 pp. (1965).
[6] A. BAR-AKIVA, In *Plant Analysis and Fertilizer Problems*, Vol. 4, p. 9. American Society Horticultural Science, Michigan (1964).
[7] E. J. HEWITT and D. G. HALLAS, *Plant Soil* **3**, 366 (1951).
[8] J. STEWART and C. D. LEONARD, *Proc. Am. Soc. Hort. Sci.* **62**, 111 (1953).
[9] E. W. BOLLE-JONES, *Plant Soil* **7**, 130 (1965).

do so. However, the *A. niger* bioassay is rather time-consuming and extremely sensitive to contamination,[10] whereas the proposed NaRA assay promises to be a simple, rapid and convenient method, as it was for nitrogen estimation.[2]

The work described in this paper is a trial to test the applicability of NaRA assay as an indicator for Mo requirements of young citrus trees in comparison with the chemical analysis and the more sensitive bioassay.

RESULTS

Mo Level Estimation

A comparison of NaRA activity with other methods as a measure of the Mo level in citrus leaf material is presented in Table 1.

Leaves were sampled from young sweet lime trees which had been supplied with gradually increasing amounts of Mo in solution culture for 8–10 weeks. NaRA assay was carried out in fresh leaf material, the bioassay and chemical analysis in the dry ashed ash solution of the same leaves.

TABLE 1. COMPARISON OF NaRA ACTIVITY WITH THE CHEMICAL ANALYSIS AND *Aspergillus niger* BIOASSAY AS A MEASURE OF Mo LEVEL IN CITRUS LEAVES
(Figures are average from twelve seedlings)

Treatment Mo in nutrient solution μg/l	NaRA activity*	Mo content, ppm of dry material	
		chemical analysis	bioassay
0	54	0·020	0·030
0·1	356	0·075	0·070
0·2	936	0·115	0·140
0·3	1104	0·115	0·140
0·4	1480	0·115	0·140
30 (control)	2532	0·180	0·250

* mμ moles NO_2 formed per 120 min per 1 g fresh material.

The results show that under conditions such as in this experiment, variations in Mo concentration of the substrate are accordingly reflected, and even amplified, in the NaRA activity of corresponding leaves.

Estimation of the Mo Requirement of Citrus Trees

It has been reported[2] that due to the inducible nature of NaRA activity regarding nitrate, it was possible to obtain information about the nitrate assimilation capacity of these leaves. According to these experiments nitrate infiltrations into these leaves has increased NaRA activity in leaves of trees low in nitrogen but not in those with a high N level. Similar experiments were carried out with Mo infiltration into the sweet lime leaves of the above mentioned solution cultures.

The results show that the low Mo-level leaves reacted to Mo infiltration with an increase in their NaRA activity. The rate of response was negatively correlated with the Mo level of these leaves, with a complete lack of response in the control leaves which were apparently

[10] D. J. D. NICHOLAS, *Methods in Enzymology*, Vol. 3, p. 1035. Academic Press, New York (1957).

in a condition of optimal or even super-optimal consumption with regard to Mo supply in the solution culture.

TABLE 2. EFFECT OF Mo INFILTRATION ON THE NaRA ACTIVITY OF Mo-DEFICIENT AND CONTROL CITRUS LEAF FRAGMENTS

Treatment Mo in nutrient μg/l	Mo, ppm in dry material*	initial NaRA activity†	NaRA activity† after Mo infiltration
0	0·020	44	536
0·1	0·050	144	578
0·2	0·080	256	440
0·4	0·080	560	792
30 (control)	0·140	792	780

* Chemical analysis.
† mμ moles NO_2 formed per 120 min per 1 g fresh material.

DISCUSSION

The results confirm that the Mo-dependent nature of NaRA enzyme may be used as an indication of the Mo level of the substrate and of the Mo content of citrus tree leaves. In this respect the measure of NaRA activity was not inferior: on the contrary, it appears to be even slightly superior to the other two methods, especially in the low Mo levels. It is true that by taking a large leaf sample and concentrating the ash solution this limitation may be overcome, but the concentration may also increase manifoldly the elements which interfere with the colorimetric chemical determination of Mo. At least in case of the spectrographic analysis of plant material, the Mo had to be omitted from the program because of the interference of calcium.[11]

According to Hewitt and Hallas[7] the sensitivity of the *A. niger* bioassay for a low level of Mo exceeds any chemical test yet devised but apparently owing to this high sensitivity it requires extreme precision in manipulation from the standpoint of contamination, spore viability, etc. In fact, our laboratory conditions, where the co-precipitation with copper sulphide is routine purification procedure for Mo-free solution cultures for citrus plants, were not sufficient for the preparation of the *A. niger* standard growth curve, which should be based not on sporulation but on mycelia yield. Apparently this is the reason why the results obtained with *A. niger* in our conditions were slightly inferior to those of NaRA assay, as a growth curve based on sporulation (number of spores) detects a concentration on the order of 10^{-3} ppm Mo, whilst mycelia yield readily detects less than 10^{-5} ppm (about $2–3 \times 10^{-6}$) and is effective at 10^{-5} ppm.[7,12,14] On the other hand, one of the great advantages of the NaRA assay described here is its simplicity, in that it does not require special skill, a trained person, or special equipment, and may be very easily adopted as a rapid field test if it is necessary.

The most outstanding feature of the proposed test lies in the results obtained with the Mo infiltration into the leaf fragments. As the rate of response to such an infiltration is negatively correlated with the Mo concentration in the substrate, it may be used as an indicator of the Mo requirement of the tested trees. If this is so, it means that the arduous task of establishing

[11] D. E. BARKER, G. W. GORSLINE, C. B. SMITH, W. I. THOMAS, W. E. CRUBE and J. L. RAGLAND, *Agr. J.* **56**, 133 (1964).
[12] E. G. MULDER, *Proc. 4th Int. Congr. Microbiol.* 489 (1947).

"leaf analysis standards" by means of years of experimentation, which is imperative for the orthodox leaf analysis[13] would not be necessary with the NaRA test. In measuring the rate of response, and not necessarily the absolute values of NaRA activity, the problems of day-to-day variations in enzyme activity due to changes in temperature, light intensity, tree condition, etc. are eliminated.

EXPERIMENTAL

Plant material. Palestine sweet lime seedlings were grown in an aerated solution culture, in a greenhouse. Purification and composition of the nutrients were according to Hewitt.[14] Ten-liter volume polyethylene buckets served as containers, one for every four seedlings. For approximately 2 months the seedlings, except those of the control treatments were deprived of Mo, and on 8 October 1965 they were divided into six groups and given 0, 0·1, 0·2, 0·3, 0·4 and 30 μg/l Mo.

Each treatment was replicated in three containers, for a total of twelve seedlings. The nutrient solutions were changed every 2–3 weeks, but the above mentioned concentrations of Mo were maintained throughout the whole experiment period. During this period the characteristic deficiency symptoms[15] developed in the 0 Mo treatment; the others differed from the control treatment only by their smaller size and stunted appearance.

Analytical procedures. Chemical analysis for Mo in the dry material of the leaves was carried out by the dithiol method described by Bingley,[16] with slight modifications. For this purpose the leaves were dry ashed in a muffle furnace at 550° and dissolved in dilute HCl. The same ash preparation was used also for the bioassay with *Aspergillus niger*, strain M. Culture solutions for the fungi were prepared according to Nicholas.[10] A standard curve for the 0–0·2 μg/50 ml solution was obtained by measuring the amount of sporulation colorimetrically[12] 100 ml water were added to culture flasks which were shaken mechanically for 5 min. Then the flasks were left standing for 15 min and finally an aliquot was taken for colorimetric reading with a blue filter (No. 42) in a Klett–Summerson colorimeter. Reliability of method was verified by spore counting.

Nitrate reductase assay is based on the method of Mulder *et al.*[4]: 250 mg fresh leaf discs, 2·5 mm diameter, incubated for 2 hr at 30° in darkness (in the light we have obtained much lower nitrite values—unpublished data), in 4 ml of 0·25 M KNO_3, adjusted to pH 7·4, with Tris buffer 0·05 M.

For measuring enzyme activity the colorimetric determination[17] of nitrite produced by these leaf fragments was used.

Introduction of Mo enzyme inducer: To the above described leaf material and incubation mixture, 1 mg Mo (as ammonium molybdate) was added and vacuum infiltrated into the leaf discs.

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[15] A. P. VANSELOW and P. DATTA NARAYAN, *Soil Sc.* **67**, 363 (1949).
[16] J. B. BINGLEY, *J. Agr. Food Chem.* **7**, 269 (1959).
[17] F. D. SNELL and C. T. SNELL, *Colorimetric Methods of Analysis*, 3rd Ed., Van Nostrand, Amsterdam (1949).