# Limitations of bioassays in macronutrient deficiency determination

**Christopher N. Guppy**[1], Neal W. Menzies[2] and Tim Harding[2]

[1]School of Rural Science and Agriculture, University of New England, Armidale, NSW 2351, Australia. Email cguppy@une.edu.au
[2]School of Land and Food Sciences, the University of Queensland, St Lucia, QLD 4067, Australia. Email n.menzies@uq.edu.au

## Abstract
Short-term nutrient bioassays can be used to assess labile nutrient availability in soils. These bioassays rely on a high number of plants and small soil volumes to exploit labile soil resources rapidly and assess potential nutrient deficiency. A comparison of the Neubauer bioassay with conventional pot trial assessment of P and S availability in a Yellow Kurosol was undertaken. Changes in labile soil nutrients and enzyme activity after bioassay assessment were also measured. The Neubauer bioassay was able to detect increased labile P availability following P fertiliser application to the soil. This corresponded with response to added P in a longer-term pot trial using maize. As expected, phosphatase activity increased following the bioassay and labile P was depleted by the plants. However, although a longer-term pot trial demonstrated the Yellow Kurosol was responsive to S fertilisation, labile S pools were sufficiently large that the short-term Neubauer bioassay detected no difference in S availability to plants. Both soil sulphatase activity and labile soil S were elevated following the bioassay. The short period of contact between the roots of the bioassay and the soil may have limited S uptake and therefore the ability of the bioassay to identify a S responsive soil. When using bioassay techniques to assess labile nutrient availability, it is critical that the size of the labile nutrient pool present be considered for each element, and that the period of contact between the bioassay and soil being tested is long enough for plant uptake to lower the nutrient supply to a level that limits further uptake.

## Key Words
Labile nutrients, nutrient availability, soil enzymes

## Introduction
The identification of nutrient deficiency in soil is an integral part of improved agronomy. The advantage of bioassays involving short-term nutrient acquisition is that a measure of labile nutrient availability is obtained without the necessity for either the large volumes of soil or relatively long periods of growth needed for pot trials. The Neubauer bioassay detects changes in labile concentrations of plant nutrients due to its attenuated nutrient acquisition biochemistry and the short period of contact between test soil and roots (Stanford and DeMent 1957; DeMent *et al.* 1959a; DeMent *et al.* 1959b). Two versions of the bioassay have been reported in the literature. Both techniques use a high seedling to soil ratio, and rely on rapid exploitation and uptake of labile nutrients during a shorter growing period (Stanford and DeMent 1957). Most authors have used a version of the Neubauer bioassay allowing plant roots access to the small volume (~100 g) of test soil for between 17 and 25 days (Deb 1969; Nair and Cottenie 1969; Nishita *et al.* 1973; Mello 1987; Grzebisz and Oertli 1992; Sattell and Morris 1992; Materechera 1999; Ortas *et al.* 1999). An alternative version of the Neubauer technique involves growing plants in sand culture without the nutrient to be tested, then transferring to the test soil for 3 days growth (Stanford and DeMent 1957; DeMent *et al.* 1959a; DeMent *et al.* 1959b). Root proliferation is rapid, and extensive ramification occurs within 24 h of contact (Stanford and DeMent 1957). As shoot growth is consistent for pre-transfer seedlings, and time of exposure is small, the amount of dry matter produced does not affect nutrient uptake and assessment (Stanford and DeMent 1957). In this paper, we examine the efficacy of the short growth period Neubauer technique (referred to as bioassay) relative to conventional pot trial assessment of nutrient availability.

## Methods
The Neubauer bioassay was used to assess the P and S status of a Yellow Kurosol soil (Isbell 1996) from Mt Cotton in south-east Queensland, Australia. This soil was known to be deficient in both nutrients. The soil was incubated with P ($KH_2PO_4$) at rates of 0, 275, 550 and 1100 mg/kg with three replicates and S ($K_2SO_4$) at 0, 137, 275, 415 and 550 mg/kg with 4 replicates for 15 days prior to the bioassay assessment and established in the glasshouse in a completely randomized design. A maize (*Zea mays*) growth pot trial was used to confirm the P and S responsiveness of the soil. The test nutrients were applied at rates of 550

 
Published on CDROM. Website www.regional.org.au/au/asssi/

mg P/kg (~100 kg P/ha) and 415 mg S/kg (~75 kg S/ha) in a completely randomized design with 4 replicates, with a basal application of all other nutrients applied. Plants were grown for 35 days.
For these studies, the Neubauer technique involved the growth of Japanese millet (*Echinocloa utilis* cv. Shirohie) seedlings in nutrient-poor river sand. To ensure consistent germination, sufficient Japanese millet seed was aerated for approximately 2 h in a 0.01 M $Ca(NO_3)_2$ solution. Wet seed (0.8 g) was spread evenly on the surface of 150 g of white river sand [Grade W7C, 0.5-0.8 mm (River Sands Pty. Ltd.)] in a 280 mL plastic container. These seeds were then covered with another 20 g of river sand and watered with 25 to 30 mL of triple deionized (TDI) water. The containers were then transferred to a controlled temperature glasshouse (23$^o$ night, 28$^o$ day) and covered with a plastic sheet to assist germination. Regular additions of nutrient solution, without any P or S depending on the nutrient to be examined, were applied from day 4 throughout the growth period (Table 1). Deionised water was also applied to maintain the plants above wilting point. Root penetration to form a thick mat on the bottom of the container was encouraged by imposing short periods of water stress.

**Table 1. Composition and timing of 2.5 mL nutrient additions to Neubauer millet (*Echinochloa utilis*) seedlings.**

| Nutrient solution | Nutrient form | Total supplied at each addition (mg) | Application days |
|---|---|---|---|
| Nitrogen | $NH_4NO_3$ | 9.0 | 4, 8, 11, 15 |
| Potassium | $KNO_3$ | 3.0 | 4, 8, 15 |
| Phosphorus | $KH_2PO_4$ | 2.0 | 4, 8, 15 |
| Calcium | $Ca(NO_3)_2.4H_2O$ | 2.0 | 4, 8, 15 |
| Magnesium | $Mg(NO_3)_2.6H_2O$ | 0.5 | 4, 8, 15 |
| Sulfur | $MgSO_4.7H_2O$ | 0.7 | 4, 8, 15 |
| Iron | Fe EDTA | 0.05 | 4, 8, 15 |

After 15 d of growth, a dense root mat had developed and the sand/plant mass was transferred to the test soil (Figure 1). Previous studies demonstrated no difference in plant response arising from changes in the growing period ranging from 11 to 19 d. Prior to transfer, the soils were brought to field capacity by the addition of nutrient solutions and TDI water to further encourage root proliferation. A slight camber in the walls of the containers permitted the sand/plant mass to be withdrawn from the original containers and placed on top of the test soil. After placement, the planted layer was tapped down to ensure good soil/root contact. Root proliferation into the test soil was rapid, with roots reaching the bottom of the test soils in 24 hours.


**Figure 1. Establishment of Neubauer method on day 15 of growth; a) incubated soil and S deficient millet (*Echinochloa utilis*) seedlings; b) dense root mat facilitating easy transfer; c) transferred seedlings. Note the pale yellow S deficiency symptoms.**

After transfer to the test soil, plants were grown for 3 d, with the soil maintained at field capacity through daily water addition. The bioassays were then harvested by removing the shoots at the level of the apical meristem. The harvested plant tops were dried to a constant weight at 60$^o$C and analysed for P or S by inductively coupled plasma atomic emission spectroscopy (ICPAES) after digestion in $HNO_3$:$HClO_4$ acid (5:1). The test soil was collected by slicing at the sand/soil interface and removing the roots by hand. Significant treatment effects were identified using the linear models function of the R statistical program (2003) with random effects conforming to a normal distribution.

Measurement of both the P and S status of the soils and enzyme activity (phosphatase and sulfatase) before and after the Neubauer bioassay was undertaken. Soil P was measured using 0.5 M $NaHCO_3$ at a 1:30 soil solution ratio and extracted for 16 hours. Following extraction, inorganic P ($P_i$) was determined using malachite green (Motomizu *et al.* 1980). Soil S was determined using the standard monocalcium phosphate extraction (Blair *et al.* 1991). Soil aryl-phosphatase activity was determined using the method of Tabatabai and Bremner (1969), whilst aryl-sulfatase activity was determined using the method of Tabatabai and Bremner (1970). Data were analysed by analysis of variance using R statistical program (2003).

**Results**

The Neubauer bioassay was able to establish a significant ($P<0.05$) increase between treatments where P had been added to the soil and the control (no P addition) but was not sensitive enough to statistically distinguish differences in P uptake at the different application rates (Table 2). For S, the bioassay did not show a statistical difference ($P>0.05$) in S availability between the control (no S addition) and any of the S rates applied (Table 2). Results are presented as shoot tissue concentrations only, as the amount of soil tested was constant and no significant difference in shoot dry matter was observed over the three days of bioassay growth (data not shown). Note that the millet seedlings were able to acquire substantial amounts of both and P and S from the test soil in three days of growth, even where no nutrient was applied (Table 2).

**Table 2. Influence of rate of P or S applied to a Yellow Kurosol on shoot tissue concentration (%) using the Neubauer bioassay. Values are the means of three replicates for P treatments and four replicates for S treatments, with standard errors in parentheses.**

| Rate of nutrient applied (mg/kg) | Shoot tissue concentration (%) | |
|---|---|---|
| | P | S |
| 0 | 0.30 (0.02)** | 0.44 (0.01) |
| 137 | - | 0.46 (0.01) |
| 275 | 0.38 (0.02) | 0.45 (0.01) |
| 415 | - | 0.45 (0.01) |
| 550 | 0.38 (0.03) | 0.46 (0.01) |
| 1100 | 0.41 (0.01) | - |
| Blank[a] | 0.22 (0.02) | 0.37 (0.02) |

** $P < 0.01$; [a]Neubauer seedlings not applied to the test soil

The response of maize to P and S addition in the 35 d pot trial, confirmed that the soil was deficient in both nutrients (Table 3). However, only tissue P concentration increased in response to fertiliser addition in the Neubauer bioassay (Table 3). No response to fertiliser S was observed (Table 3). The addition of fertiliser significantly increased the labile soil P and S pools as assessed by chemical extraction ($P<0.05$). After bioassay growth, labile soil P significantly decreased, whilst labile soil S increased ($P<0.05$) (Table 3).

**Table 3. Comparison of techniques used to assess the P and S status of an unfertilised and fertilised (415 mg nutrient/kg) Yellow Kurosol soil using both pot trial and Neubauer bioassay and effect of bioassay on soil labile nutrient pools and enzyme activity. Values are the means of three replicates for P treatments and four replicates for S treatments, with standard errors in parentheses.**

| | | P status | | S status | |
|---|---|---|---|---|---|
| | | Unfertilised | Fertilised | Unfertilised | Fertilised |
| Pot trial yield (g/pot)[a] | | 6.3 (0.5) | 20.1 (0.1) | 5.4 (0.7) | 7.1 (0.9) |
| Bioassay tissue concentration (%) | | 0.18 (0.02) | 0.26 (0.01) | 0.44 (0.01) | 0.45 (0.01) |
| Labile nutrient (mg/kg)[b] | Before[c] | 6.2 (0.3) | 67.8 (2.6) | 9.1 (0.5) | 63.6 (8.2) |
| | After | 5.0 (0.3) | 59.9 (0.3) | 17.3 (1.2) | 78.2 (1.8) |
| Enzyme activity (µg *p*-nitrophenol released/g soil/h) | Before | 500 (40) | 450 (20) | 2.7 (0.3) | 2.6 (0.5) |
| | After | 920 (60) | 860 (70) | 29.1 (1.1) | 28.3 (0.4) |

[a]P applied at 550 mg P/kg; [b]bicarbonate extractable P and phosphate extractable S; [c]measured prior to bioassay assessment (Day 15)

Plant roots may respond to nutrient deficiency through the excretion of enzymes to break down organic nutrient sources (Marschner 1995). For both nutrients, enzyme activity in the soil after the bioassay

significantly increased ($P < 0.05$; Table 3). Phosphatase activity increased by between 84 and 91%, whilst sulphatase activity increased by ~900% (Table 3). Therefore, it is likely that organic nutrient sources were mineralised and available to the millet seedlings. The increase in labile soil S after the bioassay is likely to be due to mineralisation of organic S sources and inadequate time for the millet seedlings to deplete the increased labile S pool. It is unlikely that the increased labile soil S was due to normal mineralisation over the 3 days of the bioassay rather than plant-induced mineralisation, as the sulfatase activity steadily decreased with increasing time of soil incubation (~4.6 µg *p*-nitrophenol released/g soil/h after 8 days incubation in unfertilised soil).

The Neubauer bioassay aims specifically to assess the labile pool of nutrients, and attempts this by limiting the period of contact between plant roots and soil. By increasing the size of the root sink, (large number of plants in a small volume of soil), rapid depletion of labile pool of nutrients occurs. Less labile sources of nutrient are not considered. The results presented in this paper indicate that for some elements, namely P, labile sources may be in such short supply that responses to fertiliser additions can be easily observed. Limitations in the labile P pool were also reflected in maize yield in the long-term pot trial assessment. In contrast, the bioassay results indicated that labile resources of S were adequate, yet in the long-term assessment, yield responses indicated that S was in fact a limiting nutrient in this soil. Mello (1987) reported a similar limitation in the ability of the Neubauer bioassay to detect differences in labile K availability in a highly weathered Brazilian soil, but also reported that the test had no difficulty detecting changes in labile P availability. It is therefore important to be aware of the differences in which nutrient pools bioassays are assessing. If short time periods are used, the ability of the plant to deplete the labile pool of the nutrient to be examined is critical.

**Conclusion**

The Neubauer bioassay was able to detect increased labile P availability following P fertiliser application to a Yellow Kurosol. This corresponded with a response to added P in a longer-term pot trial using maize as the test plant. However, although a longer-term pot trial demonstrated the Yellow Kurosol was responsive to S fertilisation, labile S pools were large enough that the short-term Neubauer bioassay detected no difference in S availability. When using bioassay techniques to assess labile nutrient availability, it is critical that the size of the labile nutrient pool present be considered for each element, and that the length of contact between the bioassay and soil being tested is great enough for plants to reduce the nutrient supply to a limiting level.

**References**


Blair GJ, Chinoim N, Lefroy RDB, Anderson GC, Crocker GJ (1991) A soil sulphur test for pastures and crops. *Australian Journal of Soil Research* **29**, 619-626.

Deb DL (1969) Effect of adsorbed cations and anions at different levels of saturation on the utilisation of soil and fertiliser phosphorus by crops in three Indian soils I. Neubauer experiments with rye and barley. *Plant and Soil* **30**, 227-240.

DeMent JD, Stanford G, Bradford BN (1959a) A method for measuring short-term nutrient absorption by plants: 2. Potassium. *Soil Science Society of America Proceedings* **23**, 47-50.

DeMent JD, Stanford G, Hunt CM (1959b) A method for measuring short-term nutrient absorption by plants: 3. Nitrogen. *Soil Science Society of America Proceedings* **23**, 371-374.

Grzebisz W, Oertli JJ (1992) Comparison of the Neubauer bioassay with some classical and universal extractants for determining plant available P in cultivated soils. *Communications in Soil Science and Plant Analysis* **23**, 733-746.

Isbell RF (1996) 'The Australian Soil Classification.' (CSIRO Publishing: Melbourne)

Marschner H (1995) 'Mineral Nutrition of Higher Plants.' (Academic Press Ltd: London)

Materechera SA (1999) Neubauer seedling technique to determine availability of nutrient elements for wheat from selected South African soils. *Communications in Soil Science and Plant Analysis* **30**, 2755-2767.

Mello FAF (1987) Eficiencia do metodo de Neubauer na avaliacao de fosforo e de potassio disponiveis do solo. *Revista de Agricultura* **62**, 273-279.

Motomizu S, Wakimoto T, Toei K (1980) Spectrophotometric determination of phosphate in river waters with molybdate and malachite green. *Analyst* **108**, 361-367.

Nair KPP, Cottenie A (1969) A study of the plant uptake in relation to changes in extractable amounts of native trace elements from soil profiles using the Neubauer seedling method. *Soil Science* **108**, 74-78.

Nishita H, Haug RM, Alexander GV (1973) Influence of organic matter on the availability of certain elements to barley seedlings grown by a modified Neubauer method. *Plant and Soil* **39**, 161-176.

Ortas I, Guzel N, Ibrikei H (1999) Determination of potassium and magnesium status of soils using different soil extraction procedures in the upper part of Mesopotamia (in the Harran Plain). *Communications in Soil Science and Plant Analysis* **30**, 2607-2625.

R DCT (2003) R: A language and environment for statistical computing. In. (R Foundation for Statistical Computing: Vienna, Austria)

Sattell RR, Morris RA (1992) Phosphorus fractions and availability in Sri Lankan alfisols. *Soil Science Society of America Journal* **56**, 1510-1515.

Stanford G, DeMent JD (1957) A method for measuring short-term nutrient absorption by plants: 1. Phosphorus. *Soil Science Society of America Proceedings* **21**, 612-617.

Tabatabai MA, Bremner JM (1969) Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biology and Biochemistry* **1**, 301-307.

Tabatabai MA, Bremner JM (1970) Arylsulfatase activity in soils. *Soil Science Society of America Proceedings* **34**, 225-229.