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Short communication

Staining with 0.05% neutral red reduces nutrient uptake by wheat roots

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ABSTRACT

A number of studies have used a 0.05% solution of neutral red to stain live roots so that short term root growth could be measured. These studies, which used a 5 or 10 min staining time, report no effects of the stain on plant properties such as growth, respiration, or nitrate uptake. This paper reports on two experiments conducted to determine whether this staining technique, with a 15 min stain time, affected macronutrient uptake of 6- and 7-week-old wheat (*Triticum aestivum* L.) plants grown in solution culture.

The results showed that, compared with unstained controls, staining plants with 0.05% neutral red halted or halved nitrate uptake measured over a 4 h period the following day. Potassium uptake was also significantly reduced by staining. In the experiment with smaller plants nutrient uptake rate recovered 5 days after staining, but not in the second experiment with larger plants. Stained roots were 19% narrower than unstained roots, suggesting that the stain affected the root structure. We do not recommend the use of 0.05% neutral red staining, for wheat at least, in experiments where accurate measurement of nutrient uptake rate is important.

1. Introduction

Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) is widely used as a vital stain in biology (Winckler, 1974). Although it was first proposed for animal tissues, Schumacher et al. (1983) developed a technique using it to stain plant roots so that short-term root growth could be measured. The idea was that following a staining event all newly grown root tissue would be readily distinguished as it would be unstained. Schumacher et al. (1983) reported that staining with 0.05% neutral red for 5–10 min did not affect the growth, respiration or nitrate uptake of beans (*Phaseolus vulgaris* L.) and soybeans (*Glycine max* (L.) Merr.). Their technique has been used to stain roots from a range of plant species from marshland grasses (Burdick, 1989) to succulents (North and Nobel, 1998), including cereals such as wheat (*Triticum aestivum* L.; Pritchard et al., 1987), barley (*Hordeum vulgare* L.; Lemcoff et al., 2006) and maize (*Zea mays* L.; Eticha et al., 2005). More concentrated solutions (0.15%) of neutral red have also been used (Watkin et al., 1998; Visser and Pierik, 2007). None of these studies made mention of any detrimental effect on root growth rate or diameter, although it was not the purpose of either of these studies to compare stained with unstained roots or to measure nutrient uptake rates.

A more dilute solution of neutral red (0.0002% for 1–6 h) was used to stain roots of several plant species and no detrimental effect on subsequent growth was observed (Guttenberger, 2000). Dubrovsky et al. (2006) used 0.00001–0.001% (0.4–40 μM) neutral red for time periods of up to 5 d to stain live *Arabidopsis thaliana* roots. Staining for 5 d with 0.0001% neutral red inhibited root growth, but no detrimental effect on root growth was evident when a 30 min stain time was used.

Arnold and Young (1990) stained roots of one-year-old apple seedlings with six different dyes for 15 s and recommended 1% neutral red and methylene blue above the other dyes tested because they were effective at distinguishing between new and old roots and had few adverse effects on root or shoot growth, compared with a water control. Many researchers mention that one of the reasons they use neutral red is because of its low toxicity or minimal effect on plant roots (e.g. Burdick, 1989; Guttenberger, 2000; Dubrovsky et al., 2006; Visser and Pierik, 2007).

Here we report a further test of the neutral red staining technique using 0.05% for 15 min. This was part of a larger study to understand how N and K starvation treatments affected interactions between nitrate and K uptake in wheat (e.g. Reid et al.,

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2015). The neutral red technique offered the potential to accurately measure root growth over short periods of nutrient starvation. We carried out two experiments to determine whether staining wheat roots with neutral red affected nutrient uptake rate. The focus of the research was nitrate and K, but concentrations of other macronutrients – Ca, Mg, Na, sulphate and phosphate – were also measured.

2. Materials and methods

2.1. Growth system

In both experiments, wheat seeds (*T. aestivum* L. 'Majestic') were sown on mesh on top of a 1-L container of aerated nutrient solution. These were thinned to 36 (Experiment 1) or 42 (Experiment 2) evenly sized seedlings per container. The nutrient solution was made from technical grade salts except for Fe and Cu, which were supplied as technical grade EDTA. Macro-elements were supplied in the following concentrations (mM): N as NO_3 3.75, K 3.5, P 1, S 1.9, Ca 2, Mg 1, Cl 2, Na 0.5; and trace elements (μM) as follows: Fe 45, B 46, Mn 9.2, Zn 0.8, Mo 0.2, Cu 0.4. It was necessary to include moderate concentrations of Na and Cl in the nutrient solution because these ions would be present in K-free and nitrate-free solutions (e.g. Reid et al., 2015) in proposed experiments that would use neutral red stain. Nutrient solution was initially changed weekly, with frequency increasing to thrice weekly before measurement. Plants were grown in an unheated glasshouse during winter for 6 and 7 weeks for Experiments 1 and 2, respectively. Thereafter, the following treatments were imposed:

1. Control: no neutral red staining prior to measuring uptake;
2. 20 h: roots stained 20 h prior to measuring uptake;
3. 5 d: roots stained 5 days prior to measuring uptake.

There were five replicates of each treatment. Roots were stained according to Schumacher et al. (1983), i.e. soaked in water containing a 0.05% solution of neutral red adjusted to pH 6.5; however, it was necessary to extend the soak time from 10 to 15 min to stain the large diameter roots adequately. Stain continued to leak out of the roots after staining, so the roots were washed by placement in reverse osmosis water and the water was changed ten times within 6 h, after which the roots were placed back in nutrient solution. For Experiment 1, only roots from the stained treatments were washed, but for Experiment 2 the root systems from all treatments were washed 5 days and 20 h before measurement; this was done to remove the possibility that the washing phase rather than the staining itself might have affected subsequent uptake measurements.

2.2. Nutrient uptake measurements

For the nutrient uptake measurement phase the plants were brought indoors and placed under LED growth lights at 20 °C. Plants were transferred into aerated containers filled with fresh nutrient solution (870 mL and 770 mL for Experiments 1 and 2 respectively). Samples (5 mL) of the solution around the roots were taken at 5 and 20 min, and 1, 2, 4 6, and 8 h after the solution change for Experiment 1. For Experiment 2 the first three samplings were omitted. Evaporative and sampling water losses from the solutions were replaced at each sampling time with reverse-osmosis water. The 5-mL samples were analysed for macronutrients by ion chromatography, and uptake was calculated from the decrease in nutrients relative to those in the fresh solution. The calculations included adjustments for sample removal and water added to replace evaporative losses.

After 8 h plants were harvested and shoot and root dry mass (M_d) determined. Subsamples of fresh root were taken to determine the length of stained and unstained root, using the line intersect method of Tennant (1975). Samples were placed under a microscope and root diameters were measured in three places: at the stain junction (immediately before the unstained section), 3 mm towards the root tip from the junction, and 6 mm back towards the shoot from the junction. Ten roots were measured from each replicate from the 5 d treatment. Samples were also cross-sectioned to determine which part of the root had absorbed the stain.

2.3. Statistics

The data were analysed by ANOVA (GenStat v14, VSN International 2011). Differences were deemed to be significant if $P \leq 0.05$. Raw data were used except for percentage of unstained root length, where the data were logarithmically transformed to ensure that the residuals were normally distributed.

3. Results

3.1. Plant growth

None of the staining treatments caused any significant differences in the root length, root mass, or shoot mass in either experiment (Table 1). The staining treatments resulted in a significant difference in the proportion of stained root length among treatments (Table 1), as expected. Compared with Experiment 1, plants masses in Experiment 2 were 2.5 times larger (Table 1), as a result of the extra week's growth, greater plant density (four extra plants per pot), and warmer glasshouse temperatures. As expected, in both experiments the proportion of unstained roots increased with time after the staining. The proportion of unstained root length was lower in Experiment 2, reflecting the larger pre-staining root system size.

3.2. Nutrient uptake

In Experiment 1, the time courses of solution depletion for nitrate were quite variable for the stained treatments (Fig. 1a), but suggested nitrate uptake by roots in the 20-h treatment was substantially less than those in the control and 5-d treatments. Differences in K uptake between the 20 h treatment and the other two treatments were small (Fig. 1b). In Experiment 2 the plants were larger and the depletion curves were more pronounced, showing clearly that uptake of nitrate and K was much slower in the stained treatments (Fig. 1c,d). Experiment 2 data showed no difference in uptake of nitrate or K between the 20-h and 5-d treatments (Fig. 1c,d). More nitrate-N was taken up than any other nutrient, and after 4 h the roots had depleted most of the nitrate in solution and the uptake rate slowed (Fig. 1c). This enabled some catching up by the stained treatments for times > 4 h. Therefore for both experiments we chose to compare cumulative nutrient uptake by the treatments at 4 h by ANOVA. For Experiment 1, these analyses indicated that staining 20 h beforehand significantly reduced uptake of nitrate, P and K (Table 2). For Experiment 2, cumulative uptakes of nitrate and K at 4 h were significantly less by plants in the stained treatments, particularly by those most recently stained (20-h treatment). There were small but significant differences in exudation of Cl, sulphate and Mg among the treatments in Experiment 2 (Table 2). The uptake of other nutrients was not significantly affected.