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SHORT COMMUNICATION

Quantitative determination of callose in tree roots

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Summary

The formation of callose in tree roots has been suggested as a physiological indicator of aluminum (Al) toxicity. Quantifying callose in the roots in forest soils, however, is hampered by the presence of autofluorescent materials in the roots that disturb the measurement of callose by fluorescence spectrophotometry. Tannins in the roots cause these measurement problems. Here we report on the measurement of callose in the root apices of European chestnut (*Castanea sativa*) seedlings collected in an acidified forest soil. The callose was quantified with a modified protocol which included three washing steps with polyvinylpolypyrrolidone (PVPP) before the callose was extracted. This procedure reduced the autofluorescence by about 50%. With the use of water or ethanol alone, callose could be measured in only about 15% of the root samples, whereas with the use of PVPP callose could be determined in 95% of the samples. This improved method could help to evaluate the effects of Al toxicity on tree roots grown in forest soils, where callose is detected as a physiological indicator.

Introduction

The first visible injury to plant caused by aluminum (Al) toxicity is the inhibition of root elongation (Matsumoto, 2000). However, Al-induced callose formation in the root apices of crop plants such as soybean has been suggested as an even more sensitive indicator of Al injury than the inhibition of root growth (Wissemeier et al., 1987). Callose is thus considered a powerful tool in screening for Al-sensitive or resistant cultivars (Eticha et al., 2005), although callose is naturally formed in response to wounding and pathogen infections (Kauss, 1992).

In tree roots, we recently reported that callose can serve a similar function in the root apices of Norway spruce (*Picea abies*) and European chestnut (*Castanea sativa*) seedlings exposed to Al under laboratory conditions (Hirano et al., 2004, 2006). Wissemeier

Abbreviations: CE, curdlan equivalents; FW, fresh weight; PVPP, polyvinylpolypyrrolidone

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et al. (1998) found a positive relationship between Al concentrations in the soil solution and callose concentrations in the roots of spruce collected from forest soils. However, callose quantification in field-grown tree roots is difficult because the measurement of the callose with fluorescence spectrophotometry is quenched by autofluorescent materials (Kauss, 1992; Hirano et al., 2004). Wissemeier et al. (1998) even reported measuring negative callose concentrations, indicating that the autofluorescence of the roots was higher than the callose fluorescence. One of the agents is tannic acid (Hirano et al., 2004), but other well-known tannin components, such as ellagic acid or catechin (Makker, 2003), may also play a role but have yet to be tested.

The purpose of this study was to find a procedure to reduce the autofluorescence so that the callose could be detected and quantified. This required a modification of the known protocol. For our study we used root apices of European chestnut seedlings, which are known to have a high tannin content (Heim and Frey, 2004). Additionally, we evaluated whether tannin components, such as ellagic acid or catechin, disturb the callose measurements.

Materials and methods

Tree root materials

To obtain tree roots of naturally grown plants, 20 seedlings (up to 3 years old) of European chestnut were collected from European chestnut stands in Canton Ticino in southern Switzerland. The forest soil is a podzol that has become distinctly acidified in recent decades (Blaser et al., 1999). These soils in the B horizon typically have a low base saturation ($<10\%$), a low pH (<4.8) and a kinetically restricted buffer capacity (Zimmermann et al., 2002). After collecting the seedlings, the roots were gently washed in tap water, and several 1 cm root apices were fixed in ethanol (96% (v/v)) for the callose measurement.

Callose assays comparing three washing procedures

Callose in the root apices of tree roots was assayed according to the modified method described by Hirano et al. (2004). Five milligrams (fresh weight, FW) of fixed root apices were homogenized in 2-mL reaction tubes with two steel balls (2-mm diameter) at full speed for 3 min in a swing mill (MM 2000, Retsch, Haan, Germany). To reduce the root autofluorescence, three different washing procedures were compared: three washings of the homogenized roots (i) with water (Kauss, 1992), (ii) with ethanol (20% (v/v), Hirano et al., 2004), or (iii) with polyvinylpolypyrrolidone (PVPP; Fluka No. 81385, Buchs, Switzerland; 5% (w/v) in 20% ethanol). After these steps, 1 mL of 1 M NaOH was added to the washed tissues and the tubes were heated at 80 °C for 15 min to solubilize the callose. The extract was then centrifuged for 15 min at 10,000*g* and the supernatant assayed for callose.

The callose concentration in the supernatant was quantified fluorometrically at 393 nm excitation and 484 nm emission wavelengths with a spectrofluorometer (Shimadzu RF5000, Kyoto, Japan) with the stain of 0.1% (w/v) aniline blue (water soluble, Riedel-de Haen, Seelze, Hannover, Germany) using curdlan (Sigma, Buchs, Switzerland) as a reference. Callose concentrations were expressed as μg curdlan equivalents per mg root FW (μg CE mg^{-1} FW). For each root sample, fluorescence intensities without the aniline blue stain (autofluorescence) were subtracted from the intensities in the presence of the aniline blue stain.

Effects of tannin components

To identify some of the tannin components responsible for the autofluorescence, we assayed tannic acid (Fluka), ellagic acid (Fluka), and catechin (Fluka). The concentrations of polyphenols in tree leaves including tannins in general range between 1% and 25% of total green leaf dry mass (Hättenschwiler and Vitousek, 2000), and those of tree fine roots are even higher (Gallet and Lebreton, 1995; Heim and Frey, 2004). Therefore, 25% (w/w) tannin components were used for the assay, adding 250 μg dry weight of tannin components to the tube (1 mg dry weight of roots per tube corresponds to about 5 mg FW). The fluorescence intensities with or without the aniline blue stain were recorded at 393 nm excitation and 484 nm emission wavelengths.

Statistical analyses

The fluorescence intensities and the callose concentrations were analyzed with a one-way ANOVA to identify differences among the tannin components or the washing procedures. For the significant results ($P<0.05$), the mean values of the treatments were further compared using the Tukey test. All tests were undertaken using STATISTICA '99 edition (StatSoft, Tokyo, Japan).