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Aluminium-induced effects on growth, morphogenesis and oxidative stress reactions in *in vitro* cultures of quince

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ABSTRACT

The effects of Al^{3+} [supplied as $Al_2(SO_4)_3{\cdot}18H_2O$] addition to culture media (pH 4.0) on growth, morphogenesis (in leaf explants), and oxidative stress reactions in *in vitro* cultures of 'BA 29' quince were investigated. Aluminium (Al 0.5 mM) strongly inhibited shoot growth in the proliferation and rooting phases (Al 2.2 mM), reduced shoot proliferation (Al 1.1 mM), and induced tissue browning. Superoxide dismutase (SOD) activity was increased in shoot cultures supplemented with 2 mM Al. Malondialdehyde (MDA) content of shoots was strongly increased by Al during proliferation (starting from Al 1.7 mM) and rooting (already at Al 1.1 mM), thus serving as a good 'marker' for Al toxicity. Even a low concentration of Al (0.5 mM) in the shoot induction medium was found to inhibit shoot regeneration. When standard (Al 0) shoot induction medium was used, leaf explant growth was only reduced by 2.2 mM Al in the subsequent growth phases. Following a preliminary selection for their growth on Al-enriched media, 82 potentially Al-tolerant quince somaclones were selected for further trials.

1. Introduction

The growth of many plant species is adversely affected by the presence of soluble aluminium (Al) species in soils containing high levels of organic matter and characterized by acidic reactions (pH below 5.0). However, a genetically based variability in Al sensitivity has been observed in many plants (Ghanati et al., 2005). Aluminium toxicity in acidic soils is one of the major factors reducing natural soil fertility and crop productivity, mainly in tropical and subtropical regions. In South America more than 50% of the soils present acidity problems both in surface and subsurface layers. In Brazil, in particular, fruit crop production, including pear (Rufato and De Rossi Rufato, 2008), is limited due to native soil acidity (Freire et al., 1994). In other countries, soil acidification may occur due to acid rains, cation leakage and agricultural activities (Silva et al., 2002). Surface liming acid soils may decrease Al toxicity and increase nutrient availability. However, this practice is limited in some countries for economical reasons, and cannot solve the problem of Al toxicity due to acid subsoils.

In acid mineral soils, high Al levels inhibit mineral nutrient uptake and translocation. At high H^+ concentrations, cation uptake is inhibited for two reasons: impairment of net extrusion of H^+ through the plasma membrane, and decreased loading of polyvalent cations such as Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+} in the apoplast of root cortical cells. As a consequence, uptake rates of these cations in the symplast are also lowered, and Al can accentuate this decrease, as it acts as a strongly competing polyvalent cation for binding sites in the apoplast (Marschner, 1995). Plant growth is generally reduced, and fruit quality might be negatively affected (Silva et al., 2002). Otherwise, Al can have a stimulatory growth effect on Al-accumulating plants (e.g., tea plant; Barker and Pilbeam, 2007).

Inhibition of root elongation is one of the earliest symptoms of Al toxicity in plants, which occurs within hours or even minutes of exposure to Al^{3+} (Kochian et al., 2005). There have been suggestions that Al might block plasma membrane Ca channels, therefore preventing increases in cytoplasmic Ca that are necessary for microtubule depolymerization and chromosome transport, and eventually cell expansion and divisions are negatively affected (Doncheva et al., 2005; Silva et al., 2002). The enhanced production of lateral roots in Al-treated apple plants was related to Al-induced damage to the meristematic tip of the main root, with consequent alterations in hormone production involved in apical growth (Dantas et al., 2001). Symptoms such as formation of abnormal cells in roots (Gunsé et al., 1997) and swelling of root apex (Člamporová, 2002) have been also described in plants suffering from Al toxicity. However, the primary mechanism of Al-induced inhibition of root growth is still not well-explained (Jones et al., 2006; Sun et al., 2007). The reduced capacity of the roots to explore the soil for nutrients and water can rapidly lead to severe water stress in plants (von Uexküll and Mutert, 1995).

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Aluminium ions easily bind with carboxylic and phosphate groups of plasma membranes, thus modifying their structure, lipid peroxidation then occurs and malondialdehyde (MDA) is produced as one of the end products of lipid peroxidation (Cakmak and Horst, 1991). Aluminium stress can increase the production of reactive oxygen species (ROS), and can activate in plant cells the activity of oxidative stress controlling enzymes. These include superoxide dismutase (SOD) and catalase (CAT), as a defensive mechanism to avoid damage caused by ROS (Cakmak and Horst, 1991; Giannakoula et al., 2010). Aluminium tolerance in plants can be achieved by internal (i.e., Al compartmentalization in the vacuole, detoxification by organic-acid anions) as well as external (Al immobilization at the cell wall, selective permeability of the plasma membrane, changes of the rhizosphere pH, exudation of Al-chelating compounds) mechanisms (Barceló and Poschenrieder, 2002; Piñeros et al., 2002; Kochian et al., 2005).

Aluminium has been included in several *in vitro* culture media. It was found to have no effect on plant growth at low concentrations (i.e., 30 $\mu g\,l^{-1}$ for *Prunus* meristems), and only became inhibitory at higher levels (George, 1993). The use of Al-enriched culture solutions has allowed for the selection of aluminium-tolerant variants from cultured plant cells and tissues (Van Sint Jan et al., 1997; Darkó et al., 2004; Toan et al., 2004).

Pear (*Pyrus communis* L.) cultivation is widespread in Europe and worldwide. Thus, much research has been focused on the selection of clonal rootstocks that can increase pear adaptability to environmental factors, and suitability to high density orchards (Sansavini, 2007). Quince (*Cydonia oblonga* Mill.) is one of the most widely used dwarfing pear rootstocks in Europe. It induces early bearing and high yields (Sansavini, 2007), and can easily be multiplied vegetatively by traditional methods or by micropropagation techniques (Dolcet-Sanjuan et al., 1990; Morini and Sciutti, 1991). Thus, research focused on the improvement of the regeneration processes (D'Onofrio and Morini, 2002; Marino and Berardi, 2004; Marino et al., 2008), induction of somaclonal variation and the *in vitro* selection of somaclones tolerant to abiotic stress (Bunnag et al., 1996; Marino and Molendini, 2005; Marino et al., 2000) in this species has been reported. To the best of our knowledge, there are no reports on Al toxicity on *in vitro* cultures, and on somaclonal selection for Al tolerance in quince.

Thus, the objectives of the present research are (a) to evaluate the Al sensitivity of *in vitro* cultured quince shoots; (b) to find suitable biochemical markers for Al tolerance under *in vitro* Al-stress conditions; and (c) to investigate the effect of Al on shoot regeneration from somatic tissues, and define a range of Al concentrations useful for early selection for Al tolerance at a cellular level.

2. Materials and methods

2.1. Plant material

Quince 'BA 29' was chosen due to its desirable traits as a pear rootstock. Moreover, it previously showed good adaptability to *in vitro* culture and a higher regeneration capacity than other quince clones (Fisichella et al., 2000). Two-year old donor shoot cultures of 'BA 29' were maintained (subcultures every 28–30 d of shoots obtained through axillary bud proliferation) on a standard medium (QPM, quince proliferation medium; Marino et al., 2008) with the following composition: Murashige and Skoog (1962) macro and microelements and (μM): 555 myo-inositol, 2.96 thiamine hydrochloride, 8.1 nicotinic acid, 4.9 pyridoxine hydrochloride, 26.6 glycine, 4.4 6-benzyladenine (BA), (w/v) 3% commercial sucrose (Südzucher AG, Mannheim, Germany) and 0.65% Acumedia agar (7178-01-A, Difco Diagnostic International, Milano, Italy). The pH was adjusted to 5.7 by the addition of 0.1–1N KOH before autoclaving at 120 °C for 20 min. All the chemicals were from Sigma (Sigma–Aldrich, Milano, Italy), except for sugar and agar. Standard growth conditions were: 22 ± 2 °C and a photoperiod of 16-h light (30 $\mu mol\,m^{-2}\,s^{-1}$ PAR).

2.2. Al treatments in the shoot proliferation and rooting phases

In previous trials, high Al levels associated with low pH prevented solidification of agar-enriched media; thus, liquid media were used in the present experiments. The basal composition of the proliferation media was the same as for QPM. The rooting media (QRM) differed from QPM in that they contained half-strength MS salts, but $FeSO_4$ at full strength, 2.5 μM indole-3-butyric acid (IBA), and lacked BA. Aluminium was supplied to the cultures as a sulfate salt (Van Sint Jan et al., 1997; Toan et al., 2004). The media were enriched with $Al_2(SO_4)_3{\cdot}18H_2O$ concentrations (from 0 to 740 $mg\,l^{-1}$) corresponding to 0 (control), 0.5, 1.1, 1.7 and 2.2 $mmol\,l^{-1}$ medium of total available Al. The pH was adjusted to 4.0 before autoclaving. Shoots from donor cultures were transferred to 500-ml jars (5 shoots per jar) each filled with 35 ml medium. In the proliferation phase, cotton layers (1.5 g per jar) were used as supports for the small shoots (about 10-mm long), as they could ensure adequate aeration and medium supply to the cultures, and avoid hyperhydricity that may otherwise occur after shoot submersion (Ziv, 1991a, 1991b). Perlite (Agrilit 3, Perlite Italiana, Milano, Italy) was used as a better support for longer shoots (20-mm long) in the rooting trials, and as it allowed easier removal of rooted plantlets. Jars were closed with twist-off screw metal caps, wrapped with transparent polyethylene film, and randomly placed in a growth chamber under standard conditions for proliferation, or left in darkness for the first week after transplant, for rooting. The shoot proliferation rate (pr, the number of axillary shoots obtained per initial shoot) and fresh weight (fw, the weigth of the shoot cluster obtained from each initial shoot) were recorded after 40 d in culture, without subculturing. At the end of the rooting period (total 40 d), the number of rooted shoots and the number of roots per rooted shoot were recorded. The pH of the culture media was also measured at the end of both culture phases. Shoots and root samples (quickly washed and blotted dry with towel paper) were randomly collected from different jars within each treatment, separately pooled into groups in order to have sufficient tissue amounts for biochemical analysis, dipped in liquid N_2 and stored at −80 °C until processed as reported below. Each experiment was repeated twice with shoots collected from donor cultures that had been maintained in QPM through repeated subcultures.

2.3. Al treatments during shoot regeneration

Following a time-tested standard regeneration protocol (Marino and Berardi, 2004; Marino et al., 2008), sub-apical, fully expanded immature leaves were collected from proliferating (on QPM) donor shoot cultures, 28 d after the last subculture. Three transverse incisions were made on the leaves (lacking petiole), and they were placed, with the abaxial side down, subsequentely on a shoot-induction (QSIM, quince shoot induction medium), a shoot-development (QSDM, quince shoot development medium) and a shoot-elongation (QSEM, quince shoot elongation) medium. All media were similar to QPM, but respectively enriched with (μM): thidiazuron (TDZ) 4.5 and naphthaleneacetic acid (NAA) 5.4; TDZ 4.5; BA 2.2, instead of BA 4.4. The induction phase occurred in darkness; other phases were under standard growth conditions. Each phase lasted 30 d.

2.3.1. Experiment 1

Liquid QSIM, QSDM and QSEM Al-enriched media (pH 4.0) were used. Leaf explants were cultured in 500-ml glass jars (5 leaves,