Influence of Inorganic Nitrogen and pH on the Elongation of Maize Seminal Roots

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INTRODUCTION

The below-ground environment from which plants extract nutrients and water is highly heterogeneous, both spatially and temporally. For example, inorganic nitrogen concentrations in a soil may range a 1000-fold over a distance of centimetres or over the course of hours (Bloom, 1997b). Given such heterogeneity, plants depend on various tropisms (e.g. gravitropism, thigmotropism, hydrotropism and, perhaps even, chemotropism) to guide root growth toward soil resources (Epstein and Bloom, 2005). The physiological mechanisms through which these tropisms alter growth remain uncertain, although the ‘acid growth hypothesis’ is often raised as a possibility (e.g. Chen et al., 2002; Peters, 2004).

According to the ‘acid growth hypothesis’, plants regulate cell expansion through modifying the pH around the cell wall and thereby its extensibility, which increases at low pH (Cosgrove, 1999). A large body of evidence supports this hypothesis in shoot coleoptiles or expanding leaves (Rayle and Cleland, 1992; Peters et al., 1998; Van Volkenburgh, 1999; Kotake et al., 2000; Schopfer, 2001; Friml, 2003), but results on roots have been less conclusive. Lowering the pH of the medium may either promote root elongation (Edwards and Scott, 1974; Evans, 1976; Winch and Pritchard, 1999) or have little effect (Büntemeyer et al., 1998; Peters and Felle, 1999; Walter et al., 2000). The standard approach for examining the influence of pH on cell extension has been to examine tissue segments subjected to severe treatments: for example, in Wu et al. (1996), frozen root segments were abraded with carborundum, thawed and squeezed between two glass slides to remove cell sap; in Tanimoto et al. (2000), lateral roots were killed in boiling methanol; and in Schopfer (2001), the segments were frozen, thawed, and abraded. Such treatments have been deemed necessary because the mechanical properties of cell walls in fresh, turgid tissue could be complex (D. J. Cosgrove, pers. comm.).

Rhizosphere pH changes as roots absorb and assimilate inorganic nitrogen; the assimilation of NH4+ strongly acidifies, whereas absorption of NO3− slightly alkalizes the media near the root apex (Smart and Bloom, 1998; Taylor and Bloom, 1998). These rhizosphere pH changes may be responsible for the differential patterns of root growth observed under NH4+ vs. NO3− nutrition (Bloom, 1997a; Bloom et al., 2002). Alternatively, NH4+ and NO3− themselves may be responsible for the root developmental responses (Forde, 2002).

To test the acid growth hypothesis in roots and the short-term influence of exogenous inorganic nitrogen on root elongation, a new approach was developed that provides measurements of the mechanical properties and elongation of the root apex on a real-time, continuous and non-destructive basis. Here it is reported that, in this device, exposure of a maize seminal root to a more acid medium dramatically enhanced its elasticity, but factors such as the availability of inorganic nitrogen in the medium...
had a greater influence on root elongation than cell wall mechanical properties.

MATERIALS AND METHODS

Maize (Zea mays L. cv. WF9 × Mo17) seeds were placed on germination paper (thick, fine weave, paper towelling) soaked in 1 mol m⁻³ CaSO₄ for 2 d and transferred to a 0.004 m⁻³ light-imperious polyethylene container filled with an aerated nutrient solution containing 0.15 mol m⁻³ NH₄NO₃, 1 mol m⁻³ CaSO₄, 0.5 mol m⁻³ K₂HPO₄, 0.5 mol m⁻³ KH₂PO₄, 2 mol m⁻³ MgSO₄, 0.2 kg m⁻³ Fe-NaEDTA, and micronutrients according to Epstein and Bloom (Epstein and Bloom, 2005). The containers were placed in a controlled environment chamber that provided a photosynthetic photon flux density of 400 μmol m⁻² s⁻¹ at plant height for a 14 h light period at 25°C and a 10 h dark period at 15°C.

The next day, a plant whose seminal root was 120–180 mm in length was placed into an extensiometer [for a black and white illustration of this, see fig. 3 in Bloom et al. (2002) or, for one in colour, fig. 9.5 in Epstein and Bloom (2005)]. The seedling was supported in the extensiometer by its caryopsis. The seminal root lay against a surface of the extensiometer that was tilted 4° from vertical. The side walls of the extensiometer extended outward from the surface to form a trough. A nutrient solution flowed down this trough, bathing the root. The solution contained 1 mol m⁻³ CaSO₄, 200 mmol m⁻³ KH₂PO₄ and either 100 mmol m⁻³ NH₄H₂PO₄, 100 mmol m⁻³ KNO₃, or no nitrogen, and was adjusted to pH 6.5 or 5.6 with KOH. The osmotic potential of these solutions was −0.082 MPa. Their pH was continuously monitored throughout an experiment and did not vary >0.2 pH units. At the midpoint of an experiment, 68 mOsm KCl (ψₛ = −0.14 MPa) was added to assess the response of the root elongation to a shift in osmotic potential. The end of a small plastic pipette tip was cut off, a large knot tied in one end of a nylon thread, the free end of the thread passed through the narrow opening of the pipette tip, the tip attached to the root cap with surgical-grade cyanoacrylic glue, and the other end of the thread tied to an arm connected to the shaft of a rotary variable inductance transducer (RVIT; Schaevitz 15–60, Pennsauken, NJ, USA). Weights of 1.2, 2.4, 3.6 and 5.2 g were placed on this arm to stretch the root and assess its elasticity plus plasticity and then were removed to assess elasticity alone. Applying a weight of 5.2 g was approximately equivalent to subjecting the root to an osmotic potential of −0.14 MPa [based on F = P × A, where F = force, P = pressure and A = surface area, and given that the roots had a radius of about 0.5 mm and an effective cross-section of 5% as estimated from measurements of root hydraulic conductance (Frensch and Steudle, 1989); and from micrographs of the apex (Bloom et al., 2002)]. Five minutes or longer were allotted after addition or removal of weights to permit the elongation of root apex to resume a steady rate (Fig. 1). A small piece of a wooden toothpick was glued to the root initially about 14 mm from the apex, a part of the root that is no longer elongating (Taylor and Bloom, 1998). A nylon thread connected this toothpick to a linear variable differential transducer (LVDT, Schaevitz 050 DC-D). A two-channel chart recorder logged the output from the RVIT and LVDT.

A flat-bed scanner and an image analysis program (Digitize-Pro, Dr Yaron Danon) digitized the chart recorder tracings. Apical root length was taken as the difference in the positions of RVIT and LVDT. After smoothing the data using a Gaussian kernel to compute local weighted averages, the elongation rate was calculated from the changes in length over time through numerical differentiation (Mathcad 12, Mathsoft). An FIR (finite impulse response) high-pass filter (Mathcad 12, Mathsoft) was also used to assess the sudden shifts in length when weights were added or removed. An ANOVA (General Linear Model; CoStat, CoHort Software) was used to test for significant differences among means (P < 0.05).

Neumann used a similar approach to examine the influence of NaCl (Neumann, 1993), polyethylene glycol (Chazen and Neumann, 1994) and nutrient supply (Snir and Neumann, 1997) on leaf extension, but employed a single transducer. Consequently, his extensiometer monitored the leaf as a whole and did not isolate the changes in a specific region. In the present study, to monitor the elongation of just the root apex and to eliminate any signal generated from movement of the whole plant when weights were added, the difference between two transducers was monitored.

To determine segment mass, NH₄⁺ and NO₃⁻ concentrations and osmotic potential along the maize root, individual seedling roots were exposed to the various nitrogen treatments for 18–24 h, and gently blotted dry before they were
rapidly (<2 s) frozen on a thermoelectric cold-plate mounted under a dissecting microscope. Axial sections of 1 mm length were made with a fine razor blade at 1-mm increments from 1 to 10 mm from the apex along each of ten roots. Root sections from each location were oven-dried and weighed to determine dry mass per unit length. Other root sections from each location were pooled and collected in Eppendorf tubes containing 1-5 ml of 1 mol m\(^{-3}\) CaSO\(_4\), which was adjusted to pH 3 with H\(_2\)SO\(_4\). These sections were sonicated for 30 min and then centrifuged. The supernatant was withdrawn and analysed for NH\(_4\)\(^+\) and NO\(_3\)\(^-\) as described below. There were at least three replicates for each N-treatment. Root NH\(_4\)\(^+\) and NO\(_3\)\(^-\) contents were expressed per segment water volume based on root radius measurements at each location. Two other frozen root sections from each location were immediately placed after excision into the sample chamber of a Wescor 5100 thermocouple psychrometer (Logan, UT, USA) to assess osmotic potential.

To analyse NH\(_4\)\(^+\) concentrations in the samples, a fluorimetric method based on the reaction of NH\(_4\)\(^+\) with o-phthalaldehyde (OPA) was modified (Goyal et al., 1988). An autosampler (Shimadzu Sil 9-A, Japan) injected a 50 mm\(^3\) sample into the flow of a buffer solution consisting of 126 mol m\(^{-3}\) K\(_2\)HPO\(_4\), 74 mol m\(^{-3}\) KH\(_2\)PO\(_4\), 5 mol m\(^{-3}\) OPA and 0-39 dm\(^3\) m\(^{-3}\) β-mercaptoethanol. The stream circulated for several minutes through a cabinet controlled at 64°C to optimize the NH\(_4\)\(^+\)-OPA reaction. The NH\(_4\)\(^+\)-OPA in each sample was quantified using a fluorescence detector (Shimadzu RF-551, Japan) set at 410 nm excitation and 470 nm emission. The time from sample injection to peak detection was 3-4 min with a pump flow rate of 2 cm\(^3\) min\(^{-1}\).

Analysis of NO\(_3\)\(^-\) was conducted via HPLC (Thayer and Huffaker, 1980). Samples of 50 mm\(^3\) were injected into a stream of 35 mol m\(^{-3}\) KH\(_2\)PO\(_4\) (adjusted to pH 3-0 with H\(_2\)PO\(_4\)) before passing into a 100 mm × 4-6 mm column packed with anion exchange resin (Partisil Sax 10 mol m\(^{-3}\); Whatman Laboratory, USA). The absorbance of column eluent was monitored at 210 nm. The time from sample injection to peak detection was 1-8 min.

**RESULTS**

Elongation rates of the seminal root after placing a 3-d-old maize seedling into an extensiometer equipped with two high-resolution position transducers became uniform in about 1 h (data not shown) and remained so for several hours (Fig. 1). These rates ranged from 1-5 to 2-2 mm h\(^{-1}\) (Figs 1 and 2), values similar to those reported for seminal roots of well-watered maize growing in vermiculite (Sharp et al., 1990), in solution culture (Frensch and Hsiao, 1994; Taylor and Bloom, 1998; Winch and Pritchard, 1999) or along germination paper (Bloom et al., 2002). Recovery to steady rates required <5 min after addition or removal of weights and <15 min after the addition of 68 mOsm KCl (Fig. 1). These findings indicate that the procedures used were benign and did not significantly damage the root.

Elongation of the seminal root in the nitrogen-free nutrient solution was slightly faster at pH 6-5 than at pH 5-6 (Fig. 2). Providing 100 mmol m\(^{-3}\) NH\(_4\)\(^+\) or 100 mmol m\(^{-3}\) NO\(_3\)\(^-\) in the nutrient solution at pH 6-5 stimulated elongation by 29% or 14%, respectively, in comparison with the nitrogen-free solution at the same pH (Fig. 2). The addition of 68 mOsm KCl had little effect on elongation in the nitrogen-free solutions, but decreased the rates under NH\(_4\)\(^+\) or NO\(_3\)\(^-\) by 7% and 18%, respectively (Fig. 2).

The stretching of the seminal root was proportional to the weight applied (Fig. 1) and the root fully recovered when the weights were removed (Fig. 3). This shows that the stretching response of the root apex was predominantly elastic, not plastic. Root elasticity in the more acidic solution (pH 5-6) was double that in the more neutral solution (pH 6-5). In the neutral solution, root elasticity was insensitive to nitrogen treatment (NH\(_4\)\(^+\), NO\(_3\)\(^-\) or nitrogen-free) and the presence or absence of 68 mOsm KCl (Fig. 3).

The mass of root segments were slightly higher in the plants receiving 100 mmol m\(^{-3}\) NH\(_4\)\(^+\) than in those receiving 100 mmol m\(^{-3}\) NO\(_3\)\(^-\) or in those receiving a nitrogen-free medium (Fig. 4). Concentrations of NH\(_4\)\(^+\) near the root apex were slightly higher in the plants receiving 100 mmol m\(^{-3}\) NH\(_4\)\(^+\) than in those receiving a nitrogen-free medium (Fig. 4A). By contrast, NO\(_3\)\(^-\) concentrations were negligible in the plants receiving nitrogen-free medium, but increased to over 14 mol m\(^{-3}\) in the more basal parts of the root in the plants receiving 100 mmol m\(^{-3}\) NO\(_3\)\(^-\) (Fig. 4B). The overall osmotic potential of the apex (mean ± s.e.) did not vary significantly among the nitrogen treatments...
Changes in rhizosphere pH (between pH 6 and 6.5) of apices of intact plants demonstrate that even moderate pH changes [pH 6-7] can dramatically influence the plasticity of the apex (Fig. 3), but that this may not influence the elongation of this region (Fig. 2). By contrast, the presence of exogenous ammonia stimulates root elongation (Figs 2 and 3) and accumulation of root biomass (Fig. 4) to a greater extent than that of nitrate. This is consistent with other studies (Bloom et al., 1993) and may reflect that assimilation of ammonia to glutamine consumes the equivalent of about 2 ATPs per ammonia, whereas assimilation of nitrate to glutamine consumes the equivalent of about 2 ATPs per nitrate (Bloom et al., 1992). In the carbohydrate-limited apical meristem (Bret-Harte and Silk, 1994a), the lower energy requirement for ammonia assimilation may permit cells to maintain higher elongation rates and to accumulate more biomass.

The acid growth hypothesis of roots, although well-known, has been subject to only limited experimental testing with only seven studies that have focused on this topic (Edwards and Scott, 1974; Evans, 1976; Büntemeyer et al., 1998; Peters and Felle, 1999; Winch and Pritchard, 1999; Tanimoto et al., 2000; Walter et al., 2000). Of these studies, several only examined elongation of root segments (Edwards and Scott, 1974; Büntemeyer et al., 1998; Tanimoto et al., 2000) or subjected the roots to relatively large pH changes [pH 6.5-7.0] (Evans, 1976; 7.0-7.5 (Winch and Pritchard, 1999)). The present results for root apices of intact plants demonstrate that even moderate changes in rhizosphere pH (between pH 6.5 and 5.6) can dramatically influence the plasticity of the apex (Fig. 3), but that this may not influence the elongation of this region (Fig. 2). By contrast, the presence of exogenous inorganic nitrogen significantly enhanced elongation (Fig. 2), even though the seedlings had ample nitrogen reserves in the caryopsis to support growth for several more days (Bloom et al., 2002).

The meristem and transition zones (Baluska et al., 1996) near the root apex differ from more mature root zones in that they lack fully differentiated phloem tissue. Import of carbohydrates or some nutrients (e.g. K+) from more mature tissues into the root apex may depend on diffusion through the symplast (Bret-Harte and Silk, 1994b) or may be enhanced by pressure driven flow from sieve tube elements (Boyer and Silk, 2004; Gould et al., 2004). Nevertheless, little of the nitrogen absorbed in the maturation zones moves toward the apex (Siebrecht et al., 1995; Walter et al., 2003); therefore, the nitrogen required for cell division and isotropic cell expansion may derive primarily from nitrogen that the apical zones themselves absorb and assimilate.

The stimulation of root elongation in the presence of exogenous ammonia or nitrate is an appropriate response with ecological implications (Bloom, 1997b). Plants predominantly obtain nitrogen through root absorption of ammonia and nitrate from the soil solution (Bloom, 1997b). Spatial and temporal availability of soil inorganic nitrogen is highly heterogeneous, as mentioned above. To survive under such heterogeneity and under competition from soil microorganisms, plant roots must be in the right place at the right time. Appropriately, roots proliferate in soil regions that are nitrogen-rich (Hackett, 1972; Drew, 1975; Grime et al., 1986; Sattelmacher and Thoms, 1989; Bingham et al., 1997; Robinson et al., 1999; Zhang et al., 1999). Specifically, proliferation of lateral roots seems critical for exploiting nitrogen-rich regions (Bloom et al., 2002; Forde, 2002). The phenomenon observed here—acceleration of seminal root elongation by exogenous inorganic nitrogen—would rapidly position the mature zones of seminal roots, from where lateral roots emerge, adjacent to nitrogen-rich soil regions.

The presence of ammonia stimulated root elongation (Figs 2 and 3) and accumulation of root biomass (Fig. 4) to a greater extent than that of nitrate. This is consistent with other studies (Bloom et al., 1993) and may reflect that assimilation of ammonia to glutamine consumes the equivalent of about 2 ATPs per ammonia, whereas assimilation of nitrate to glutamine consumes the equivalent of about 12 ATPs per nitrate (Bloom et al., 1992). In the carbohydrate-limited apical meristem (Bret-Harte and Silk, 1994a), the lower energy requirement for ammonia assimilation may permit cells to maintain higher elongation rates and to accumulate more biomass.

Diminishing the osmotic potential of the nutrient solution from -0.08 to -0.22 MPa by the addition of 68 mOsm KCl had no effect in the nitrogen-free treatments, but depressed root elongation under nitrate and nitrite nutrition (Fig. 2). In maize, apical zones of the root rapidly absorbed ammonia and nitrate (Taylor and Bloom, 1998). Most of the ammonia absorbed promptly disappeared from the tissues (Fig. 4A), presumably, as it was assimilated into amino acids (Bloom et al., 2002). Some of these amino acids may serve as metabolically benign osmotolites to support cell expansion in the elongation zone (Rhodes et al., 2002). By contrast, a portion of the nitrate absorbed remained as free nitrate within the apical zones (Fig. 4B), providing another metabolically benign osmolyte (up to 29 mOsm or -0.063 MPa) to
support expansion (Bloom, 1996; Bloom, 1997a; McIntyre, 2001). The addition of 68 mOsm KCl to the nutrient solution depressed root elongation possibly because it counteracted the osmotic effects of the stored amino acids and NO₃⁻. Although the osmotic potential resulting from the amino acids, NO₃⁻ or KCl was small in comparison to the total osmotic potential of the apex (1-1 MPa), these metabolically benign osmolytes were probably not distributed evenly throughout the root, but concentrated in the more metabolically active compartments (Aspinall and Paleg, 1981).

It is unlikely that the effects of 68 mOsm KCl on the plants receiving NH₄⁺ or NO₃⁻ were specific to the ions K⁺ and Cl⁻. The high-affinity transport systems for NH₄⁺ and NO₃⁻, which predominate at 100 mmol m⁻³, are insensitive to the presence of K⁺ (Scherer et al., 1984; Bloom and Finazzo, 1986; Smart and Bloom, 1988; Bloom and Sukrapanna, 1990) or Cl⁻ (Glass et al., 1985; Bloom and Finazzo, 1986; Deane-Drummond, 1986). In previous experiments on maize roots, mannitol and KCl were indistinguishable in their effects on cell turgor and root elongation (Frensch and Hsiao, 1995). Nonetheless, it was found that roots of intact plants exhibited simple mechanical properties; elongation was linear with the strain applied (Fig. 1) and recovery from the strain proceeded with the strain applied (Fig. 1) and recovery from the strain was complete (Fig. 3). These results indicate that cell wall properties alone do not regulate root elongation.

Some researchers have questioned whether cell wall mechanical properties can be measured on live, turgid tissue because walls suffering both strain in all directions due to turgor and strain in a unidirectional vector due to the addition of weights might behave in a complex manner. Nonetheless, it was found that roots of intact plants exhibited simple mechanical properties; elongation was linear with the strain applied (Fig. 1) and recovery from the strain was complete (Fig. 3). These results permit a simple interpretation.

Proton pumps in the apical zones of roots generate what are known as ‘growth currents’ that have an important role in the determination and regulation of root polarity (Weisenseel et al., 1979; Miller and Gow, 1989). A previous
study (Taylor and Bloom, 1998) found that maize roots exposed to either 100 mmol m\(^{-3}\) NH\(_4\)\(^+\), 100 mmol m\(^{-3}\) NO\(_3\)\(^-\), or nitrogen-free media pumped sufficient protons to maintain the root surface in the elongation zone at least 0-4 pH units more acidic than the bulk solution; the greatest pH differentials, however, were in the nitrogen-free treatment. These results indicate that the differences in root elongation observed in the current study were not simply that the various nitrogen treatments produced different pHs in the elongation zone.

Walter et al. (2003) reported that elongation of maize roots was faster in pure water than in a nutrient solution containing both NH\(_4\)\(^+\) and NO\(_3\)\(^-\), a finding contradictory to those presented here. This solution, however, provided NH\(_4\)\(^+\) at 3-85 mol m\(^{-3}\), a concentration several times higher than the highest levels measured in agricultural fields in Davis, California (Jackson and Bloom, 1990), and the maize roots grown in this solution accumulated high levels of free NH\(_4\)\(^+\) (40 \(\mu\)mol g\(^{-1}\)) in the meristems. For comparison, in the present study, the nutrient solution used contained 0-1 mol m\(^{-3}\) NH\(_4\)\(^+\) and free NH\(_4\)\(^+\) concentrations in the root meristems were <6-7 \(\mu\)mol g\(^{-1}\) (Fig. 4A, calculated on the basis that root segments were 87 % water). High accumulations of free NH\(_4\)\(^+\) in tissues are toxic because they dissipate pH gradients in mitochondria and plastids (Epstein and Bloom, 2005). Thus, NH\(_4\)\(^+\) toxicity might explain the differences between the results of the two studies.

In summary, the results of the present study indicate that in well-watered maize plants, exogenous inorganic nitrogen more than pH or cell wall elasticity or plasticity influences the elongation of the root apex.

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