Growth Response of *Eutrema salsugineum* Seedlings on Media Containing Different Levels of Potassium

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ABSTRACT

Potassium (K^+) is a macronutrient essential for optimum plant growth and yield. Despite its agricultural significance, farmers frequently overlook the benefits of K^+ fertilizers so K^+ deficiencies can develop in crops. A defined nutrient medium was devised to assess the response of seedlings of the extremophile crucifer, *Eutrema salsugineum* (Yukon and Shandong accessions), to conditions lacking added K^+ (0 mM K^+) or K^+ at concentrations of 0.5 and 2.0 mM. *Arabidopsis thaliana* seedlings were also grown to provide a comparison to a well-documented phenotype for K^+ deficiency symptoms. Due to different growth rates, the experiment ended on Days 10 and 15 for Yukon and Shandong *Eutrema*, respectively, compared to Day 7 for *Arabidopsis*. Like *Arabidopsis*, *Eutrema* shoot and root biomass was reduced in seedlings grown on media lacking K^+ relative to plants on 2 mM K^+ plates but the reduction in root biomass was most pronounced for *Arabidopsis* (5-fold) compared to *Eutrema* (2-fold). *Eutrema* had significantly shorter primary roots on 0 mM K^+ plates and the 1.8-fold decrease relative to Yukon seedlings on 2 mM K^+ plates was similar to the 2-fold reduction for Shandong plants. *Eutrema* seedlings had significantly reduced lateral root growth on 0 mM K^+ plates although the 1.8-fold reduction relative to seedlings on 2 mM K^+ plates was not as great as the 7-fold decrease for *Arabidopsis* roots. Shandong seedlings did not show K^+-specific changes in root hair morphology but root hairs of Yukon seedlings were over 2-fold longer on 0 mM K^+ plates compared to those of roots on 0.5 mM or 2 mM K^+ plates. Overall, the data shows that *Eutrema* seedlings cope well with low K^+ conditions but there are accession-specific differences that distinguish Yukon from Shandong plants.

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ABBREVIATIONS

µg	Microgram(s)
µL	Microlitre(s)
µM	Micromolar
µmol	Micromole
AKT1	*Arabidopsis* potassium transporter 1
ANOVA	Analysis of Variance
C	Celsius
CBL	Calcineurin B-Like Proteins
CIPK	CBL-Interacting Protein Kinase 23
cm	Centimeter(s)
d	Day(s)
DW	Dry Weight
g	Gram(s)
h	Hour(s)
HAK5	Potassium high-affinity transporter 5
K^+	Potassium
KCl	Potassium Chloride
kg	Kilogram(s)
L	Litre(s)
M	Molar
m	Metre(s)
mM	Millimolar
mmol	Millimole

min	Minute(s)
mL	Millilitre
N	Nitrogen
Na^+	Sodium
P	Phosphorous
ppm	Parts Per Million
ROS	Reactive Oxygen Species
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
s	Second(s)
TCA	Tricarboxylic Acid Cycle

LITERATURE REVIEW

I. Crop Requirements for Potassium (K^+)

Potassium (K^+) is an essential macronutrient and the most abundant cation present in plant cells accounting for up to 10% of total plant dry weight (Leigh & Jones, 1984). Not surprisingly, plants have high requirements for K^+ from their environment (Leigh & Jones, 1984). When crops experience K^+ deficiency their ability to withstand environmental stressors such as drought, cold, or attack by pathogens and pests is impaired (Armengaud et al., 2004; Amtmann et al., 2008). Crops have a high demand for K^+ throughout the growing season that often peaks during specific developmental stages. For example, the amount of K^+ taken up by winter wheat reaches a maximum of 200 kg K^+ per hectare at the flowering stage (Mengel, 1980). In the case of potatoes, K^+ uptake in the whole plant steadily increases throughout the growing season and reaches a maximum of well over 400 kg K^+ per hectare (Potash Development Association, 2011). The extremely high "peak requirements" of K^+ that many crops experience illustrate the importance of maintaining soil K^+ at levels that prevent a deficiency from developing and maximizes crop yield.

Typical soil K^+ levels range from 0.1 to 1 mM (Fageria, 2009). Unfortunately, each time a crop is harvested the K^+ acquired by the plant from the soil during its growth is removed. Without K^+ replacement, crop removal of K^+ eventually decreases soil fertility (Mengel, 1980). Methods of replenishing K^+ on agricultural land include the application of manure and crop residues to fields. However, due to the high amount of K^+

removed when crops are harvested, additional K^+ fertilization is often required (IPI, 2012).

II. Potash Fertilizers

K^+ fertilizers, collectively known as *potash*, are used to replenish agricultural soils (IPI, 2012; Potash Development Association, 2011). The most common form of potash fertilizer used by farmers is "muriate of potash" where K^+ is in the chemical form of potassium chloride (KCl) (Potash and Phosphate Institute, 1998). Other forms of potash include sulphate potash (K_2SO_4), nitrate potash (KNO_3), and mono-potassium phosphate potash (KH_2PO_4) (IPI, 2012; Potash and Phosphate Institute, 1998).

Despite the fact that crops require high levels of soil K^+, the application of K^+ fertilizers is often neglected (Pandey et al., 2007). Of particular concern is the fact that potash fertilizers are frequently applied at very low doses compared to other macronutrients like nitrogen (N) or phosphorous (P) (IPI, 2012; Mengel, 1980). That is, when applied at frequently used rates, potash often only replenishes about 35% of the K^+ removed from the soil during harvest (Smil, 1999). Poor restoration of K^+ content has decreased soil K^+ concentrations in farmlands all over the world. For example, approximately 75% of the rice fields in China are reported to be K^+ deficient and the same is true for over 60% of the wheat fields in Australia (Römheld & Kirkby, 2010). The increasing production of biofuels from crop residues has further reduced the rate at which K^+ is being returned to the soil (Smil, 1999). When crops do not receive adequate supplies of K^+ during the growing season, particularly during times of peak uptake

requirements, yield decreases and economic losses are inevitable (Ebelhar & Varsa, 2000; Sweeney et al., 2001; Harris, 1997).

III. Food Production and Economics

As the global population increases, so too does the requirement for increased food production. However, poor soil nutrient conditions have serious adverse consequences on crop yield (Smil, 1999; IPI, 2012; Pandey et al., 2007). A perfect example of this occurred in 1995 in the cotton fields of Georgia, USA when farmers discovered a new leaf-spot disease that affected nearly 2000 acres of cotton across the state, and by 1996, the disease was found in 20 000 acres (Harris, 1997; Wrona, 1996). Diseased plants had characteristic brown spots that were caused by the fungal pathogen *Stemphylium solani* and the disease was called *Stemphylium* leaf-spot. Application of fungicide sprays did not help alleviate the problem. As scientists attempted to pinpoint the cause of this disease, soil testing revealed one common factor between the majority of the infected fields tested: K^+ deficiency (Harris, 1997). The lack of K^+ in the crop compromised the strength of the cotton plants and increased their susceptibility to fungal infection. This resulted in yield losses from 100 to 400 pounds per acre during the 1996 season (Wrona, 1996). In contrast, the benefit of adding K^+ is clearly demonstrated by studies showing yield increases of 6 to 10% in wheat fields when K^+ fertilizers are applied (Ebelhar & Varsa, 2000; Sweeney et al., 2001). For corn, as soil K^+ levels increased to around 250 parts per million (ppm), yields increased by approximately 190 bushels per acre (Potash and Phosphate Institute, 1998). As discussed above, there is, nonetheless, a general

reluctance to apply sufficient K^+ to crops that can be directly attributed to potash supply and fluctuations in its cost (IPI, 2012; Gulati, 2014).

A recent report released by TD Economics outlines how global potash prices have historically been extremely variable and that even over the last two years potash prices have risen and fallen drastically (Gulati, 2014). Farmers are confronted with expensive and fluctuating potash supplies that result in an unwillingness to risk purchasing K^+ fertilizers. As a management cost, farmers receive less profit for their product when fertilizers increase in price. Moreover, global fertilizer subsidies influence fertilizer use in developing nations like India where subsidies currently favor N-based fertilizers (Armengaud et al., 2009).

With only a finite area of arable farmland available on our planet, increasing soil productivity and crop yield is of paramount importance. With this challenge looming, a greater mechanistic understanding of how K^+ deficiency impacts plant productivity and how plants take up and assimilate K^+ are important research topics that can help select traits to develop crops that show improved efficiencies in their use of K^+.

IV. K^+ in Plants

K^+ is critical for many cellular functions including osmotic balance, activation of enzymes involved in photosynthesis and respiration, for protein synthesis, starch synthesis, stomatal movements, and the transport of sugars, water, and nutrients (Maathuis & Sanders, 1996; Wang & Wu, 2010). Cellular functions requiring K^+ can be divided into two broad categories: 1) processes that require stable, localized concentrations of K^+ in cellular compartments and 2) processes that rely on the high

mobility of K^+ ions and their movement between cellular compartments (Armengaud et al., 2009; Pilot et al., 2003). In general, stable K^+ concentrations are associated with metabolically active compartments such as the cytosol or mitochondrial matrix (Armengaud et al., 2009). Functions requiring K^+ in these subcellular compartments typically include enzyme activation and protein synthesis (Armengaud et al., 2009). In contrast, plant processes that rely upon the high mobility of K^+ include stomatal opening and closing and phloem transport (Pilot et al., 2003).

V. K^+ Sensing at the Root Surface

K^+ uptake from the soil is described as a "biphasic" process involving a high affinity system (mechanism I) and a low affinity system (mechanism II) (Epstein et al., 1963). When soil K^+ concentrations fall below 20 μM, K^+ uptake is mediated by an active process (mechanism I) and when soil concentrations are above 200 μM, plants acquire K^+ passively (mechanism II). The complementary operation of high and low affinity uptake systems at different K^+ levels enable plants to adapt to variations in K^+ supply (Epstein et al., 1963). High affinity K^+ uptake is thought to be inducible by stress attributed to low K^+. However, the signaling mechanism(s) involved in this low K^+ stress response are unknown (Hirsch et al., 1998; Pandey et al., 2007).

Plants contain several voltage-gated channels that are divided into groups based on their voltage dependence. Inward rectifying channels are mainly responsible for K^+ uptake (Liu et al., 2013). These types of channels are activated when the root plasma membrane becomes hyperpolarized or "more negative". Hyperpolarization of the plasma membrane is one of the first major events that occur during a K^+ deficiency (Xu et al.,

2006; Liu et al., 2013). A well-known inward rectifying K^+ channel called *Arabidopsis* potassium transporter 1 (AKT1) helps mediate sustained root K^+ uptake (Xu et al., 2006). This channel is activated by the interaction of the protein kinase CBL-Interacting Protein Kinase 23 (CIPK23) with the calcium sensors Calcineurin B-Like Proteins 1 or 9 (CBL1/9). This complex, commonly known as a CIPK-CBL complex, activates AKT1 through phosphorylation resulting in K^+ uptake by the root (Xu et al., 2006; Liu et al., 2013). The channel is open or closed in response to the activation potential (voltage difference) across a membrane and is activated under hyperpolarization conditions (Liu et al., 2013).

Research with the *Arabidopsis* knock-out mutant *akt1* has demonstrated the importance of AKT1 activity during K^+ deficiency in that *akt1* plants had severely diminished growth on low-K^+ media ($\leq$ 100 μM) compared to wild-type plants grown under the same conditions (Hirsch et al., 1998). This channel may be vital in K^+ sensing at the root surface (Hirsch et al., 1998; Pandey et al., 2007) based upon the fact that: (1) it is active during both high and low affinity K^+ uptake, (2) it can alter its K^+ uptake kinetics based on external K^+ concentrations, and (3) *akt1* mutants do not show the initial plasma membrane hyperpolarization response to K^+ deficiency, evidence consistent that a defect in AKT1 renders plants defective in establishing a membrane potential (Hirsch et al., 1998).

VI. Plant K^+ Acquisition from Soil

K^+ acquisition by plants is determined by two main factors: (1) the amount of K^+ mobilized to the surface of roots through a combination of diffusion and mass flow and (2) root K^+ uptake kinetics (Kolahchi & Jalali, 2006; Fageria, 2009).

Factor 1: Amount of soil K^+ and root surface

Most soils typically contain 0.3 to 25 grams of K^+ per kilogram of soil (Fageria, 2009). Topsoil contains the bulk of K^+ available to crop plants but the majority of K^+ in the soil, roughly 90 to 98%, is unavailable for plant uptake (White & Greenwood, 2013). The bulk of soil K^+ is actually contained in minerals such as feldspars or micas and it is only released through very slow weathering processes. Soil K^+ available to crops is contained in one of three separate K^+ pools: (1) K^+ in soil solution, (2) exchangeable K^+ associated with negatively charged mineral sites or organic matter, and (3) non-exchangeable K^+ (also termed "slowly" exchangeable K^+) attached to clay lattices (Kolahchi & Jalali, 2006; White & Greenwood, 2013). The rate at which K^+ is exchanged between these pools largely determines K^+ availability at any given time as K^+ in the soil solution is actively replaced by exchangeable K^+. K^+ in the soil solution is the most readily available for plant uptake (Kolahchi & Jalali, 2006; White & Greenwood, 2013). The ability of soil to retain K^+ largely depends on the amount of clay and organic matter present, since these negatively charged sites, termed "cation exchange sites," hold K^+ in place. Typically K^+ does not leach from soil unless the sand content is high and the clay or organic content is low because these types of soils do not contain high amounts of cation exchange sites (Kolahchi & Jalali, 2006).

Factor 2: K^+ transporters

The distribution of K^+ within plant cells varies between cellular compartments. In the cytosol, healthy plant cells have stable K^+ concentrations around 100 mM whereas vacuolar K^+ stores can range from 10 to 200 mM (Fageria, 2009). Despite the fact that plants require these high levels of cellular K^+ for normal growth, typical soil K^+ concentrations range from just 0.1 to 1 mM (Fageria, 2009) requiring that K^+ be taken up by root cells against a concentration gradient. As a consequence, plants have evolved efficient K^+ transport systems that maximize soil K^+ uptake by roots.

The potassium high-affinity transporter 5 (HAK5) is reported to be one of the most important K^+ transport proteins involved in high affinity K^+ uptake in *Arabidopsis,* accounting for an estimated 50% of K^+ uptake during K^+ stress (Aleman et al., 2008; Qi et al., 2008). *Arabidopsis* seedlings exposed to K^+ deficient conditions showed a significant up-regulation of this transporter (Gierth et al, 2005; Aleman et al, 2008). In fact, *AtHAK5* is the most strongly up-regulated gene in K^+-deficient *Arabidopsis* plants (Qi et al., 2008; Gierth et al, 2005; Aleman et al, 2008). Due to its highly inducible response to K^+ deficiency, *HAK5* expression has been used as a bio-marker for monitoring K^+ stress in *Arabidopsis* (Gierth et al, 2005).

VII. K^+ Deficiency Symptoms

There are numerous environmental factors that can limit K^+ availability to plants. Conditions such as drought, density of soil, or high sodium (Na^+) levels can result in periods of K^+ starvation that negatively impact plant growth (Aleman et al., 2008; Desbrosses et al., 2003). Symptoms of K^+ deficiency include reduced growth, chlorosis

that often leads to necrosis at leaf tips and/or margins, slim and weakened stems, decreased lateral root growth, and an overall loss of crop yield (Maathuis & Sanders, 1996; Kim et al., 2010; Leigh & Jones, 1984). K^+-starved plants also exhibit disruption in osmotic potentials, reduced photosynthetic rates due to the requirement of K^+ as a counter-ion for proton-flux across thylakoid membranes, reduced transport of sugars, and impaired transpiration regulation due to perturbed guard cell function (Armengaud et al., 2009; Gierth et al., 2005).

Plants experiencing K^+ deficiency are also more susceptible to various biotic and abiotic stresses. For example, researchers at the International Potash Institute reviewed the findings of nearly 2000 independent studies investigating the benefits of K^+ nutrition on disease and pest resistance (Perrnoud, 1990). In this meta-analysis, 70% of studies describing plants exposed to fungal or bacterial pathogens showed that plants had improved resistance when given adequate K^+ and 63% of studies on mite and insect injury showed an association between decreased predation with improved K^+ nutrition (Perrnoud, 1990).

VIII. K^+ Deficiency and Enzyme Activation

As stated earlier in this section, maintenance of cytosolic K^+ concentrations is absolutely crucial for the proper function of many enzymes in plant cells. Approximately 60 enzymes require K^+ as a cofactor and many of them are involved in the key metabolic processes of nitrogen or sugar metabolism (Jones & Pollard, 1983). Some well known K^+-dependent enzymes include pyruvate kinase, Rubisco, nitrate reductase, and starch synthase (Jones & Pollard, 1983; Armengaud et al., 2009).

Typically, a brief exposure of plants to K^+ deficiency does not significantly alter cytosolic K^+ levels because vacuolar K^+ stores can counteract minor fluctuations (Jones & Pollard, 1983). However, if a K^+ deficiency persists, cytosolic K^+ concentrations eventually decrease and this negatively impacts proper enzyme activation and function (Kim et al., 2010). Armengaud et al. (2004) reported that exposure of A*rabidopsis* seedlings to prolonged K^+ deficiency resulted in noticeable primary and secondary metabolite changes. For example, K^+ deficient seedlings had significantly higher levels of soluble carbohydrates (60 - 65% higher for sucrose and 50 - 53% higher for glucose) but considerably lower levels of both nitrate (24% lower) in the roots and acidic amino acids in root and shoot tissue (27 - 46% for glutamate and 32 - 42% for aspartate). It has been theorized that the inhibition of pyruvate kinase in the roots during prolonged K^+ starvation is a direct cause of these changes in metabolite levels leading to an inhibition of glycolysis (Armengaud et al., 2009). Interestingly, many of these changes in metabolite concentrations were partially reversed within 24 h after K^+ was resupplied to the growth medium and these reversible changes coincided with a reversible change in the activity of several enzymes including those involved in glycolysis, TCA cycle, sugar metabolism and nitrogen assimilation (Armengaud et al., 2009).

IX. Root Architecture and K^+ Deficiency

Given that plants cannot change location, they must adapt to any adverse changes in their environment in order to survive (Lynch, 1995). Changes in the spatial arrangement of roots (termed root architecture) are well-studied traits in plants because root development is responsive to environmental cues (Shin & Schachtman, 2004; Jung et

al., 2009; Kim et al., 2010). Plants can alter their root growth to maximize the absorptive surface area of their roots in soil that, in turn, optimizes nutrient uptake. Nutrient deficiencies elicit distinct responses in root architecture and these responses can vary significantly depending on which nutrient is in short supply (Lynch, 1995; Kim et al., 2010; Jain et al., 2009).

A classic K^+ deficiency phenotype that has been widely reported in *Arabidopsis* seedlings is a reduction in lateral root proliferation (Shin & Schachtman, 2004; Jung et al., 2009; Kim et al., 2010). K^+-starved *Arabidopsis* seedlings show a decrease in lateral root length as well as a decrease in the number of lateral roots. Interestingly, studies on the impact of K^+-starvation on primary root growth have had conflicting results. Shin and Schachtman (2004) reported no impact on primary root growth but Jung et al. (2009) and Kim et al. (2010) reported a reduction in primary root growth with K^+ deficiency. Overall, plants undergo a decrease in root biomass when exposed to prolonged periods of K^+ deficiency. This reduction in biomass is not surprising since K^+ is required in large quantities for turgor-driven cell expansion (Jung et al., 2009; Kim et al., 2010).

The impact of K^+ on root morphology has also been studied. Root morphology differs from root architecture because it refers to the specific characteristics of a single root (i.e. root diameter or root hair growth) (Lynch, 1995). *Arabidopsis* seedlings exposed to periods of K^+ deficiency show a lengthening of root hairs. Desbrosses et al. (2003) measured the root hair length of 3-day-old *Arabidopsis* seedlings grown in the presence of 0, 10, 50 and 100 mM KCl and found that root hairs became longer with decreasing K^+ content. The promotion of root hair growth during K^+ deficiency serves to

increase root surface area in the soil and maximize K^+ uptake. Shin & Schachtman (2004) reported that root hairs of K^+-deprived *Arabidopsis* seedlings increase their production of the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2). This increase in ROS is directly dependent on ethylene signaling and ethylene has been implicated in the response to low-K^+. Jung et al. (2009) demonstrated through the use of ethylene inhibitors that *HAK5* expression responds positively to ethylene production during K^+ deficiency and that ethylene may act upstream of ROS. By blocking the accumulation of ethylene, *Arabidopsis* ethylene mutants did not accumulate ROS in their roots, *HAK5* expression did not increase, and plants became hypersensitive to low K^+. ROS-mediated root hair elongation may therefore comprise a K^+ deficiency signaling mechanism although sensors that initiate this response have not been identified (Desbrosses et al, 2003; Shin & Schachtman, 2004; Jung et al., 2009).

X. Studying Mineral Deficiencies In Agar-Grown Seedlings

One of the biggest challenges facing research into mineral deficiencies in plants is establishing and maintaining a deficient environment for the duration of the experiment. The use of agar culture has been a simple and effective method to study changes in root architecture while depriving seedlings of specific ions but it can be very difficult to create and sustain long-term nutrient deficiencies (Gruber et al., 2013). Unlike hydroponics, a system whereby roots of plants are suspended and continuously aerated in aseptic liquid culture, agar-grown plants require the use of gelling agents to solidify the growth medium. Commonly used gelling agents for plant culture have included agar (a mixture of the polysaccharide agarose and agaropectin extracted from the cell walls of red algae)

and Phytagel (a water-soluble polysaccharide made of glucuronic acid, rhamnose, and glucose that is produced by the fermentation of the bacteria *Sphingomonas elodea*) (Gruber et al., 2013). Unfortunately, different types of gelling agents contain varying levels of elemental contaminates so the type of gelling agent selected for nutrient studies depends largely on the nutrient of interest but choosing the correct agent is of upmost importance. Gel selection has been shown to significantly impact the morphological responses of plants to specific nutrient deficiencies and this can result in misleading or conflicting conclusions (Gruber et al, 2013; Jain et al., 2009). For example, P deficiency has been extensively studied in *Arabidopsis* seedlings grown on agar media. Jain et al. (2009) reported that different types of agar contained varying levels of P and this contamination can adversely impact the P deficiency response of seedlings. Gruber et al (2013) concluded that agar choice is a problem that is not limited to studies of P but to research on other macro- and micronutrients as well. In fact, they reported that there is no single gelling agent that is satisfactory for every type of plant nutritional study (Gruber et al., 2013).

There are currently many different types of gelling agents commercially available for plant culture. For example, Sigma-Aldrich has over 40 different types of agar products available for plant and animal culture research (Sigma-Aldrich Co.). Unfortunately, papers often fail to include the specific type of agar used or the lot number. This missing information can present a significant challenge in replicating research as studies have shown that different types of agars, including different batches of the same agar, contain varying levels of contaminants making documenting gel media

used and their lot numbers very important (Jain et al., 2009; Gruber et al., 2013). For example, Jain et al. (2009) found significant differences in the amount of P and iron (Fe) present in different batches of Sigma agar. In addition to quality concerns, complications include the availability of the agar type used or discontinuation of agars reported in earlier studies. For example, Gruber et al. (2013) outlined different *Arabidopsis* root architecture elicited by nutrient deficiencies on various media. In their study they used Duchefa agar (Duchefa Biochemie, The Netherlands) for K^+ deficiency studies in *Arabidopsis* but it is not currently available in Canada. In summary, the selection of the appropriate gelling agent is crucial in order to obtain deficiency results that are consistent and reproducible between and within laboratories.

XI. *Eutrema salsugineum* and nutrient stress

Although extensive research has been done with the model plant *Arabidopsis* and its response to K^+ deficiency, less work has been done with its close relative *Eutrema salsugineum.* *Eutrema* shows a higher tolerance to certain stressors (i.e. salt and cold stress: Gong et al, 2005; Griffith et al., 2007) and has been reported to show a greater tolerance to low nitrogen conditions (Guevara et al. 2012; Kant et al., 2008). Many agricultural lands have experienced increased salinization and decreased nutrient levels due to improper land management (Römheld & Kirkby, 2010). The gradual degradation of available farmland makes the study of extremophile plants an attractive approach to identifying traits associated with plant tolerance to environmental stress.

XII. Hypothesis and Research Objectives

In this thesis, I hypothesized that *Eutrema salsugineum* would show comparable K^+ deficiency symptoms to those already confirmed for its close relative *Arabidopsis thaliana.* K^+-deficiency phenotypes for *Arabidopsis* seedlings grown on defined nutrient media include an inhibition of both primary root and lateral root length (Shin & Schachtman, 2004; Jung et al., 2009; Kim et al., 2010). For my study, I compared two *Eutrema* accessions that have been frequently used including one from China (Shandong Province) and one from Canada (Yukon Territory) (Amtmann, 2009). In order to test the hypothesis that the two species would behave similarly under K^+ deficient conditions, several experimental objectives needed to be met: (1) design a plate experiment protocol that reproducibly exposes seedlings to K^+ deficient conditions, (2) quantitatively analyze root architecture, (3) compare root architecture of plants grown on K^+-sufficient and K^+-deficient media, and (4) analyze the K^+ ion content of plants grown on K^+-sufficient and K^+-deficient media.

MATERIALS AND METHODS

I. Seed information

Arabidopsis thaliana seeds (Ecotype Col-0) were bulked from seeds originally obtained from the *Arabidopsis* Biological Resource Centre at Ohio State University (https://abrc.osu.edu). *Eutrema salsugineum* seeds of the Yukon accession were originally collected from plants in the Yukon Territory, Canada, and seeds were bulked for use at McMaster University. In this thesis, the single seed descent line designated Tsh5 S5 was used (Champigny et al, 2013). Seeds of the Shandong accession were progeny from seeds originally obtained from Dr. Ray Bressan, Purdue University. Shandong seeds were from the single seed descent line produced by our lab and designated Th2a S5. All seeds were stored in the dark at room temperature in 1.5 mL Sarstedt microfuge tubes (Cat. No. 72.690).

II. Chemicals and Reagents

Unless otherwise specified, all chemicals and reagents were purchased from Sigma Aldrich, Oakville, Ontario. All reagents and media were prepared with H_2O that was deionized using a Barnstead^{TM} NANOpure II water filtration unit (Thermo Fisher Scientific Inc, USA) and then sterilized by autoclaving when specified. Three gelling agents were used in this study: Sigma Phytagel (Cat. No. P8169, Batch No. SLBB5682V) Sigma Agar (Cat. No. A1296, Batch No. SLBF1721V) and Difco Noble agar (Cat. No. 0142-01). The gel agent used is specified where necessary.

III. Seed Sterilization

Approximately 20 µL of seeds were transferred to a 1.5 mL microfuge tube. Shandong seeds often required closer to 30 µL due to lower germination frequency. Seeds were surface sterilized with 1 mL of 70% ethanol, gently shaken for 2 min, then the ethanol was removed and discarded. A sterilization solution was made that contained 50% Javex® bleach (commercial grade diluted with H_2O), and 0.1% (v/v) Triton-X100 detergent. One mL of this sterilization solution was added, the tubes were gently shaken for 10 min, then the solution was removed and discarded. Seeds were then rinsed by adding 1 mL of autoclaved H_2O, inverting the tube 2 to 3 times, then removing and discarding the liquid. This rinse step was repeated 5 to 7 times until there were no signs of foam left. Seeds were then re-suspended in 1 mL of 0.1% autoclaved agar and stratified in the dark for 24 h at 4°C.

IV. Germination Conditions

Seeds were germinated on round 100 x 20 mm tissue culture dishes purchased from Sarstedt (Cat. No. 83.1802.003, Sarstedt Inc., Newton, NC). Germination media contained media buffered with 3 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and solidified with 0.8% Difco noble agar (w/v) and adjusted to pH 5.7. The media was then autoclaved and plates were poured in a laminar flow bench. Once solidified, plates were inverted, sealed in plastic bags, and stored at 4°C until use. Sterilized seeds suspended in agar were dispensed onto MES media plates with a P200 Pipetman (Gilson Inc., Middleton, WI). Seeds were placed 5 mm apart to allow room for germination and then sealed with ½ inch Micropore™ tape (Cat. no. 1530-0: 3M Health Care, St. Paul, MN).

The plates were placed in a Conviron growth cabinet (Model no. A1000) set to 23°C with 24 h light photoperiod (60 μmol m^{-2} s^{-1}) until the radicle just emerged from the seed coat, approximately 2.5 to 3 d. *Eutrema* seeds take longer to germinate than *Arabidopsis* seeds so *Eutrema* seeds were plated on MES plates 24 h before *Arabidopsis* seeds to synchronize their transfer to nutrient agar plates.

V. Nutrient Media Plates

a) Preparation of 100 x 15 mm Square Plates:

After germinating, seedlings were transferred to sterile nutrient medium in 100 x 15 mm square polystyrene BD Falcon™ Integrid plates (Cat. No. 351112). Nutrient plates contained variable K^+ ranging from 0 mM K^+ to 2 mM K^+. When necessary, KNO_3 additions were replaced by variable additions of 0.5 mM $NaNO_3$ to reduce or exclude K^+ additions to the media contributed by KNO_3. The composition of the nutrient plates is given in Table 1. The medium also contained 2% (w/v) sucrose and was solidified with 0.8% (w/v) agar. The pH was adjusted to between pH 5.5 to 6 with 5 M NaOH. To transfer seedlings from the MES germination plate to the nutrient agar plate, curved jewelers forceps from Fischer Scientific (Cat. No. 08953F) were used to hook the radicle from beneath and gently lift the seedling off of the media. Each nutrient plate contained 6 seedlings aligned evenly on the second gridline from the top of the plate and spaced approximately 13 mm apart. Seedlings were placed on the second gridline from the top of the plate in order to give shoot tissue adequate room to grow. Transplanting was done in a laminar flow bench and all tools used were sterilized with 70% ethanol and forceps were flame-sterilized.

Table 1. Components for Agar "Square" Plates

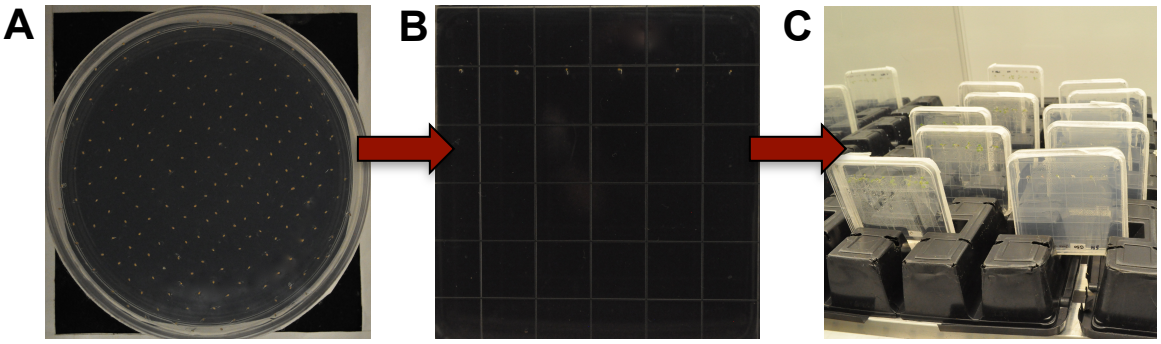
Component	Stock	Final	Potassium Concentration (mM)		
			2	**0.5**	**0**
Macronutrients	M	mM	Volume per 1 L final volume (mL)		
$CaCl_2 \cdot 2H_2O$	0.5	0.5	1	1	1
$MgSO_4$	0.5	0.5	1	1	1
KNO_3	0.5	0.5	4	1	0
$NaNO_3$	0.5	0.5	0	3	4
$Na\text{-}PO_4$*	0.5	0.5	1	1	1
Micronutrients	mM	μM			
$FeSO_4 \cdot 7H_2O$	10	100	10	10	10
H_3BO_3	100	100	1	1	1
$MnCI_2$	20	20	1	1	1
$CuSO_4$	1	1	1	1	1
$ZnSO_4 \cdot 7H_2O$	3	3	1	1	1
$NaMoO_4 \cdot 4H_2O$	1	0.4	0.4	0.4	0.4
$CoCI_2$	0.1	0.01	0.1	0.1	0.1
H_2O			978.5	978.5	978.5

*Prepared from a mix of 0.5 M Na_2PO_4 and 0.5 M NaH_2PO_4 solutions to yield stock of pH 7

The plates were sealed with Micropore^{TM} tape and then positioned vertically in a Conviron growth chamber (Model No. PGC20) set at 23°C under a 12 h light cycle with illumination set at 170 $\mu mol^{-1}m^{-2}\ s^{-1}$.

Photographs of plates were taken at the same time each day using a Nikon D90 camera (Nikon Corporation, Japan) fitted with a Nikon DX 18-55 mm lens. Root measurements were obtained from the digital images using the program ImageJ (Wayne Rasband, National Institutes of Health, USA, version 1.46r). Primary root length, lateral root number, lateral root density, total root length, and total lateral root length were measured. After 10 d, seedlings were harvested, root and shoot tissue was separated and the biomass for each was determined. Tissue was then flash-frozen in liquid nitrogen for long-term storage at -80°C. A general overview of the experiment is shown in Figure 1.

Figure 1 - Schematic overview of nutrient plate experiment protocol. (A) Seeds germinating on MES media, (B) seedlings on the day of transfer, and (C) plates positioned vertically in the growth chamber.


A
B
C

b) Preparation of 245 x 245 mm Square Plates

Seedlings were transplanted onto sterile nutrient medium in 245 x 245 mm square polystyrene Corning plates (Cat. No. 07 200 134; Corning Incorporated, NY), containing medium with variable K^+ composition prepared as described above and outlined in Table 1. Each nutrient plate held 6 to 9 seedlings, aligned evenly on the second gridline from the top of the plate and spaced approximately 12.5 to 25 mm apart. Growth conditions and seedling root and shoot measurements were performed as described for the 100 X 15 mm grid plates above. Samples were flash-frozen in liquid nitrogen for long-term storage at -80° C.

Photographs of plates were taken at the same time each day using a Nikon D90 camera (Nikon Corporation, Japan). Root measurements were obtained from the digital images using ImageJ. Primary root length, lateral root number, lateral root density, total root length and total lateral root length were measured. After 10 d, the seedlings were harvested, root and shoot tissue was separated and the biomass for each was determined. Tissue was then flash frozen in liquid nitrogen for long term storage at -80°C.

VI. K^+ Content Determination

Leaf or root tissue stored at -80° C was transferred to a 1 mL NextGen^{TM} V glass vial (Cat. No. 986254; Wheaton, NJ). A small amount of liquid nitrogen was added to each vial and tissue was ground using a polypropylene pellet pestle (Sigma Cat. No. Z359947). Each sample contained pooled leaf or root material from one nutrient plate representing approximately 2 to 10 mg fresh mass of tissue. Typically, shoot tissue yielded more fresh mass than root tissue. Tissue mass was recorded and the opening of

the vial was covered with a small square of cheesecloth secured to the vial with a rubber band. Vials were then placed on a styrofoam holder and partially submerged in liquid nitrogen for 30 s. The vials were then immediately freeze-dried in a 6 L FreeZone freeze dry system (Labconco) for 24 h. Once the samples were completely dry, sample dry weight (DW) was recorded. To each vial, 1 mL of 1 M HNO_3 was added to hydrolyze the tissue and a solid top phenolic cap (Cat. No. W986254NG, Wheaton) was used to secure the vial. Samples were vortexed for 30 s and then the vials were incubated at 70°C for 24 h. Samples were then diluted to a concentration range of 1 to 100 µM using 0.2 % (w/v) CsCl and K^+ content was determined using a Varian SpectrAA 220 FS (Varian Inc.). For root tissue, dilutions typically ranged from 10X to 20X. For shoot tissue, dilutions ranged from 40X to 80X for 2 mM K^+ and 0.5 mM K^+ plates, and 10X to 20X for 0 mM K^+ plates. Before samples were tested for K^+ content, a standard curve of known K^+ concentrations was done to calibrate the machine with five points making the standard curve ranging from 0 mM K^+ to 100 mM K^+ (Fig. 2). Standard solutions were made by diluting KCl in deionized H_2O.

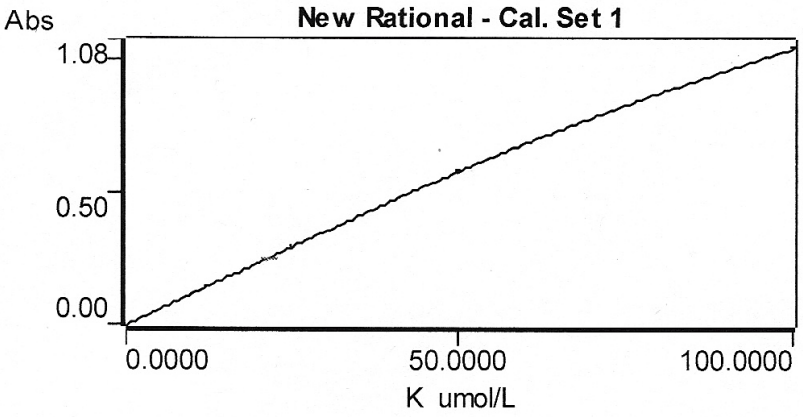
VII. Microscopy

Pictures of root hairs were done using a Leica stereomicroscope (Model M165 FC, Leica Microsystems Inc, ON, Canada). Leica Application Suite imaging software (version 4.3) was used to obtain high-resolution images.

Figure 2 - Standard Curve of known K^+ concentrations for flame atomic absorption spectrometry.

Method: K (Flame)

Sample ID	Conc umol/L	%RSD	Mean Abs	Readings		
CAL ZERO	0.0000	2.3	0.0298	0.0306	0.0296	0.0293
STANDARD 1	12.5000	1.2	0.1437	0.1456	0.1433	0.1423
STANDARD 2	25.0000	0.9	0.2958	0.2927	0.2972	0.2973
STANDARD 3	50.0000	0.3	0.5760	0.5775	0.5746	0.5759
STANDARD 4	100.000	0.4	1.0365	1.0313	1.0391	1.0393



VIII. Statistical treatment of data

Statistical analysis was done using the programming software RStudio™ (RStudio Inc, © 2009-2012, version 0.97.248). Results of the five daily root measurements were individually examined for each of the three K^+ treatments for each species: primary root length, lateral root number, lateral root density, total lateral root length and total root length. Root and shoot fresh weights were also analyzed in a similar fashion.

Data was first compiled into a comma separated values (.csv) file in order for the data to be compatible with the RStudio software. Before any statistical analysis was done, the data underwent a Box-Cox transformation in order to find the simplest transformation that resulted in normality. This analysis showed that no transformation was necessary so all data used for statistical analysis in this thesis used linear (untransformed) data. Box-cox transformations were done using the following code:

```
(boxcox(RootParmeter)~Treatment, data = mydata)
```

Once normality was established, a Type I analysis of variance (ANOVA) was done individually for each root parameter for each species to determine if the data was significant between the three K^+ treatments. Type I ANOVA tests were done using the following code:

```
anova(lm(RootParameter~Treatment, data=mydata))
```

Significant results underwent a post-hoc test in order to compare the means. Post-hoc tests were done using the following lines of code:

```
example<-aov(RootParameter~Treatment, data=mydata)
LSD.test(example, "Treatment", group=TRUE)
```

RESULTS

I. Effect of agar choice on K^+ nutrient response

Various studies investigating K^+ nutrition in *Arabidopsis* seedlings have used different types of gelling agents to generate a K^+ deficiency. Since Phytagel was already regularly used in this lab with success for the study of other nutrient deficiencies, it seemed a logical first choice to analyze *Eutrema* seedlings exposed to varying K^+ conditions.

a) K^+ deficiency studies of seedlings grown on media solidified with Phytagel

Figure 3A shows representative seedlings on Day 7 following transfer to Phytagel-based nutrient medium. The seedlings were growing equally well on all of the K^+ concentrations tested including the plate with no added K^+. The noteworthy symptoms of K^+ deficiency reported in the literature, including inhibition of lateral root growth and chlorosis of shoots, were not detected.

Five root parameters were measured daily to describe seedlings grown on media solidified with Phytagel and the data for Day 4 is summarized in Figures 3B, C, D, E and F. The results show no statistically significant indications that root development was altered for any of the seedlings grown on K^+ deficient media or when K^+ was added. However, there was a significant difference observed between the responses of the plants used in this study in that Shandong seedlings had fewer lateral roots, lower lateral root density, and shorter lateral roots relative to Yukon seedlings based upon the measurements of lateral root growth taken on Days 4 (Fig. 3C, D, E and F) and Day 7, the

Figure 3 – Response of *Eutrema salsugineum* and *Arabidopsis thaliana* seedlings to varying K^+ on Day 4 using media solidified with Phytagel. (A) Representative four-day-old Shandong *Eutrema* (*Eutrema* (S)), Yukon *Eutrema* (*Eutrema* (Y)), and *Arabidopsis* seedlings. Average measurements of (B) primary root length, (C) number of lateral roots, (D) lateral root density, (E) total lateral root length, and (F) total root length. Data shown are the mean ± SE of 2 biological replicates (*n* = a minimum of 34 seedlings). A one-way ANOVA was used to test for significance between treatments and data was found not to be significant ($P > 0.05$).

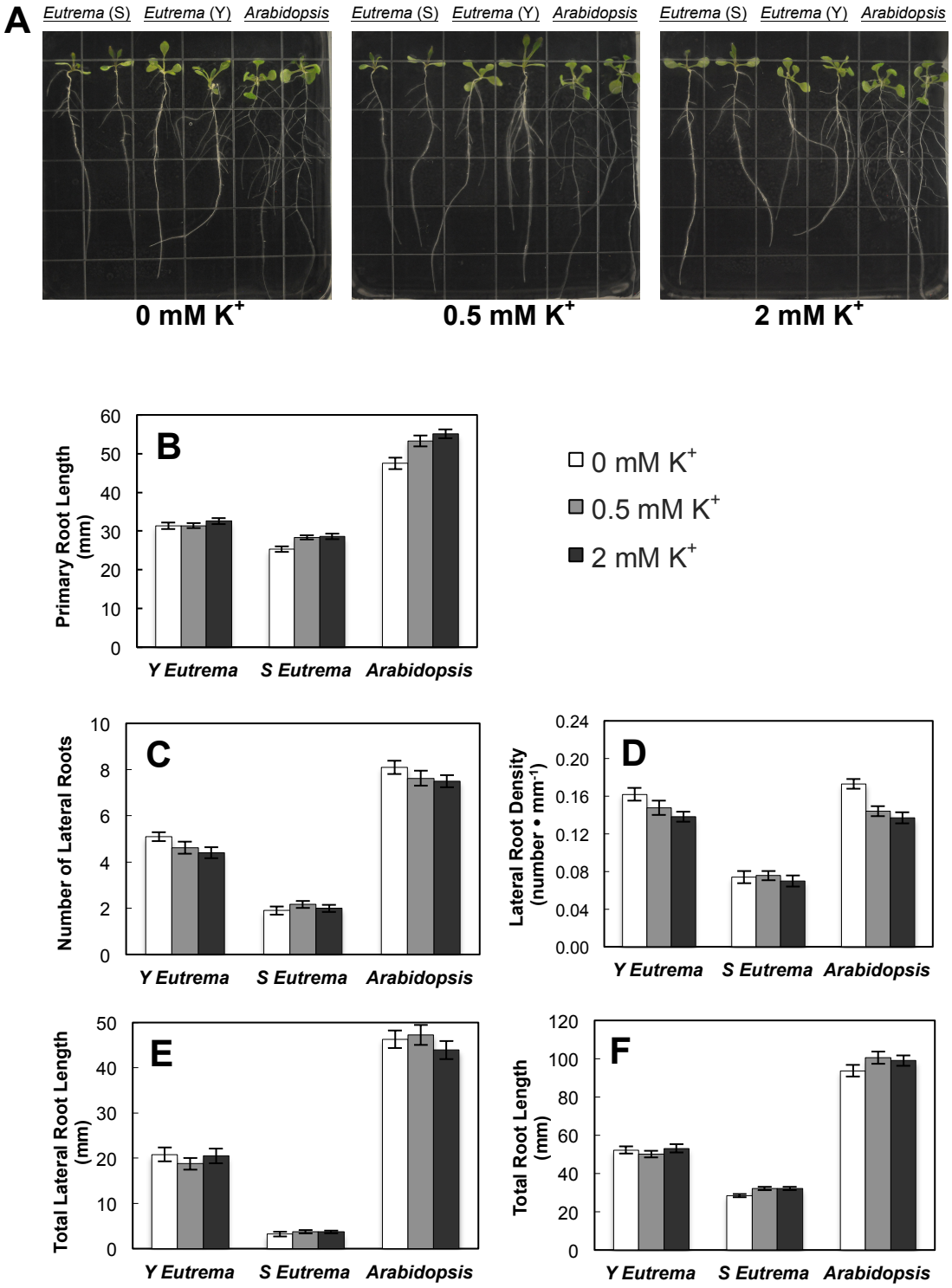
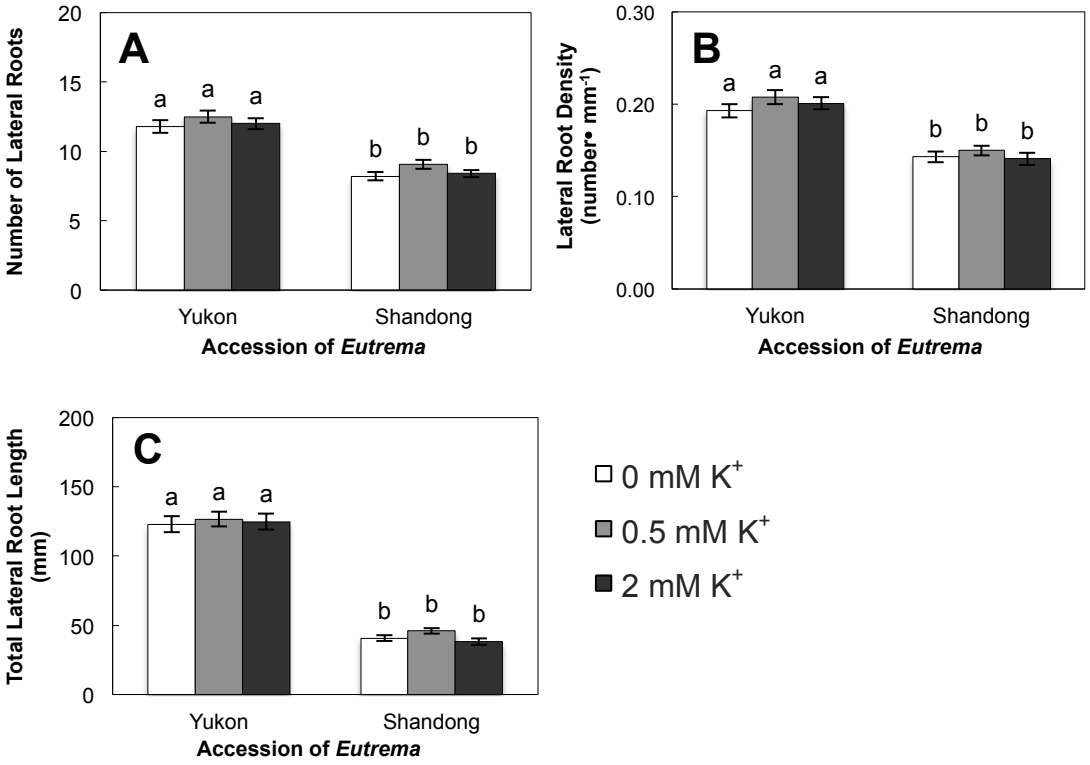

A
Eutrema (S) Eutrema (Y) Arabidopsis
Eutrema (S) Eutrema (Y) Arabidopsis
Eutrema (S) Eutrema (Y) Arabidopsis
0 mM K+
0.5 mM K+
2 mM K+
B
Primary Root Length (mm)
0 mM K+
0.5 mM K+
2 mM K+
Y Eutrema S Eutrema Arabidopsis
C
Number of Lateral Roots
Y Eutrema S Eutrema Arabidopsis
D
Lateral Root Density (number • mm-1)
Y Eutrema S Eutrema Arabidopsis
E
Total Lateral Root Length (mm)
Y Eutrema S Eutrema Arabidopsis
F
Total Root Length (mm)
Y Eutrema S Eutrema Arabidopsis

Figure 4 – Response of Yukon and Shandong *Eutrema salsugineum* seedlings to varying K^+ on Day 7 using media solidified with Phytagel. Average measurements of seedlings on Day 7 with respect to (A) number of lateral roots, (B) lateral root density, and (C) total lateral root length. Data are the means of 2 biological replicates ± SE (*n* = a minimum of 34 seedlings per species per treatment). Means were analyzed by a two-way ANOVA and were significant between the two accessions ($P < 0.05$) but were not significant between the 3 treatments ($P > 0.05$). For each panel, the bars designated by the same letter are not significantly different ($P > 0.05$).


A
Number of Lateral Roots
20
15
10
5
0
a a a
b b b
Yukon
Shandong
Accession of Eutrema
B
Lateral Root Density (number• mm-1)
0.30
0.20
0.10
0.00
a a a
b b b
Yukon
Shandong
Accession of Eutrema
C
Total Lateral Root Length (mm)
200
150
100
50
0
a a a
b b b
Yukon
Shandong
Accession of Eutrema
0 mM K+
0.5 mM K+
2 mM K+

final day of the experimental period (Fig. 4A, B and C). *Arabidopsis* seedling roots were too long to accurately measure on Day 7 so these seedlings grew faster than both *Eutrema* accessions under the same growth cabinet conditions.

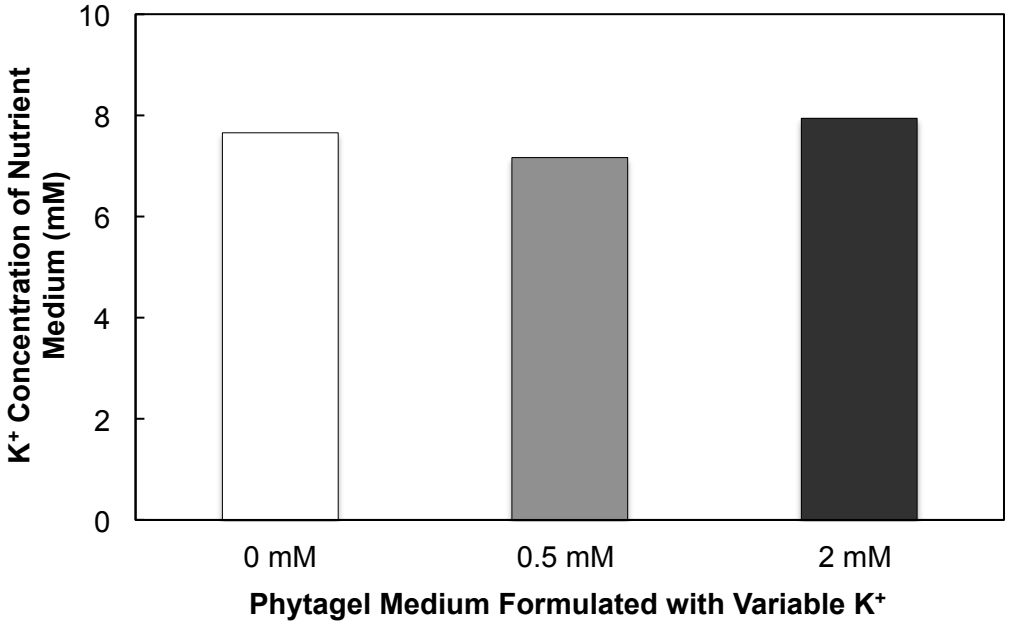
To summarize the results, we found no evidence that seedlings grown on nutrient plates solidified with Phytagel exhibited a characteristic K^+ deficiency. This was especially evident when looking at lateral root measurements, a frequently used symptom of K^+ deficiency (Shin & Schachtman, 2004; Jung et al., 2009; Kim et al., 2010). However, the growth response on Phytagel for the three plants tested differed with Shandong plants showing the least number and shortest lateral roots compared to Yukon *Eutrema* and *Arabidopsis*.

b) Phytagel use in K^+ deficiency experiments

The lack of a K^+-responsive change in root phenotype observed for the seedlings from the three plant sources (Fig. 3 and 4) strongly suggests that the seedlings were not deficient with respect to K^+. If this was the case, then the seedlings must have had access to K^+ even when no K^+ was added implicating the gel and/or components used to prepare the gel as providing sufficient K^+ to meet the seedling requirements for this essential macronutrient.

The K^+ analysis of the Phytagel nutrient plates is given in Figure 5 and the data indicates that there was no significant treatment-specific difference in K^+ content of the nutrient plates as all of the plates had approximately 7 mM K^+. This result is evidence that K^+ was being delivered by one of the formulation ingredients, namely the gelling agent (Phytagel) and/or the chemical stock solutions (Table 1). Moreover, the K^+

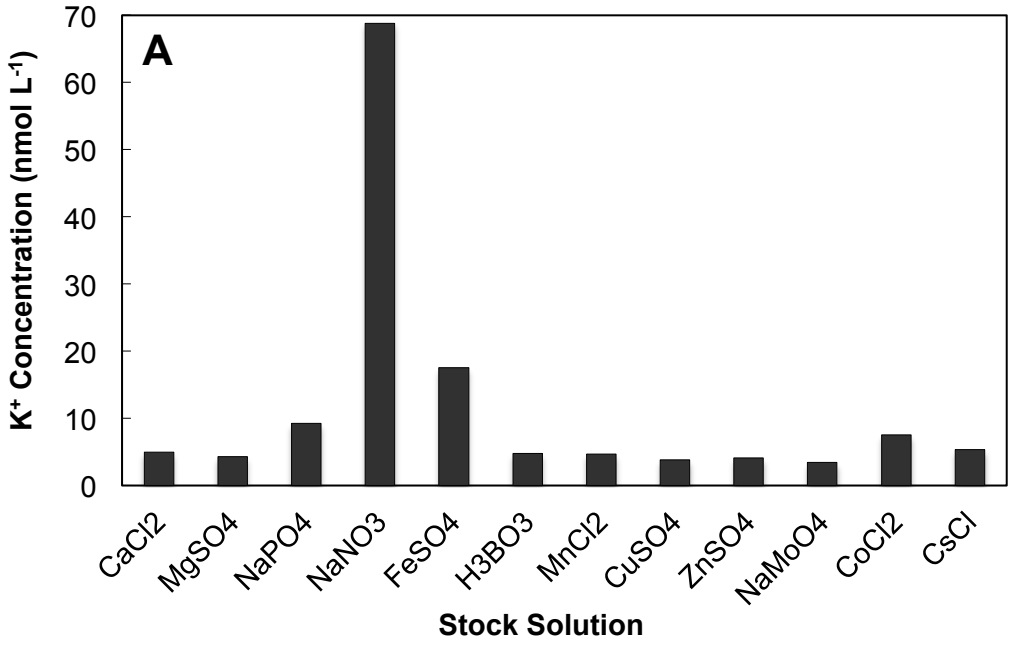
Figure 5 - K^+ content of Phytagel medium used for K^+ nutrition experiments. Media tested was formulated to contain one of three K^+ concentrations with all other nutrients unchanged (see Table 1 for composition). K^+ was determined by atomic absorption spectrometry. For this analysis, an approximate 250 mg square plug was removed from the gel matrix of a defined nutrient plate and processed for K^+ content following the protocol outlined for tissue in the Materials and Methods section. Data represents a single determination but the experiment was repeated 3 times with similar results.


10
8
6
4
2
0
K+ Concentration of Nutrient Medium (mM)
0 mM
0.5 mM
2 mM
Phytagel Medium Formulated with Variable K+

concentration in the medium was approximately 3.5-fold higher than the highest intended target deficiency level of 2 mM K^+. To discern the source of K^+, the K^+ content of the macro- and micronutrient stock solutions was determined and the results of this analysis indicate that macro- and micronutrient stock solutions only contained trace amounts of K^+ (Fig. 6A) and, as such, cannot be the major source of external K^+. For example, the stock with the highest K^+ concentration was $NaNO_3$ and it would take over a L to deliver 70 nmol K^+ to the nutrient gel. This left Phytagel as the prime candidate for K^+ in the nutrient test plates. The K^+ content of Phytagel was compared to two sources of agar (Nobel and Sigma) and Phytagel was found to contain approximately 300-fold more K^+ than the two sources of agar tested (Fig. 6B).

When we realized that Phytagel contained such high amounts of contaminating K^+ we looked at the K^+ content of some of the seedlings grown on these plates and selected 7-day-old *Arabidopsis* seedling roots for K^+ content analysis. The K^+ content of 7-day-old *Arabidopsis* seedlings as a function of dry tissue mass is shown in Figure 7. This data indicates that there is no significant difference in the K^+ content of seedlings grown on nutrient plates solidified with Phytagel regardless of whether K^+ is added to the gel media (2 or 0.5 mM K^+) or not (0 mM K^+). In summary, the elevated K^+ content present in Phytagel produced nutrient plates that were almost invariable with respect to K^+ concentration and yielded seedlings that had similar levels of this macronutrient. As a result, all subsequent results describing the impact of K^+ on seedling growth are experiments carried out with Sigma agar (Cat. No. A1296, Batch No. SLBF1721V) as the gelling agent. Figure 8 outlines the differences in growth patterns that occur when agar is

Figure 6 - K^+ content of stock solutions used to make the defined nutrient plates. (A) K^+ content of macro- and micronutrient stock solutions (see Table 1, Materials and Methods) expressed in nmol per L (nM). Each sample contained 500 μL of the stock solution of interest and 500 μL CsCl. (B) K^+ content of granular Phytagel and agar gelling agents used for nutrient deficiency studies. K^+ contents are expressed in mM. For each sample, K^+ was extracted from 11 mg of gelling agent using 1 M HNO_3 and then diluted using CsCl. Results are the means of 3 biological replicates ± SE.


A
70
60
50
40
30
20
10
0
K+ Concentration (nmol L-1)
CaCl2
MgSO4
NaPO4
NaNO3
FeSO4
H3BO3
MnCl2
CuSO4
ZnSO4
NaMoO4
CoCl2
CsCl
Stock Solution

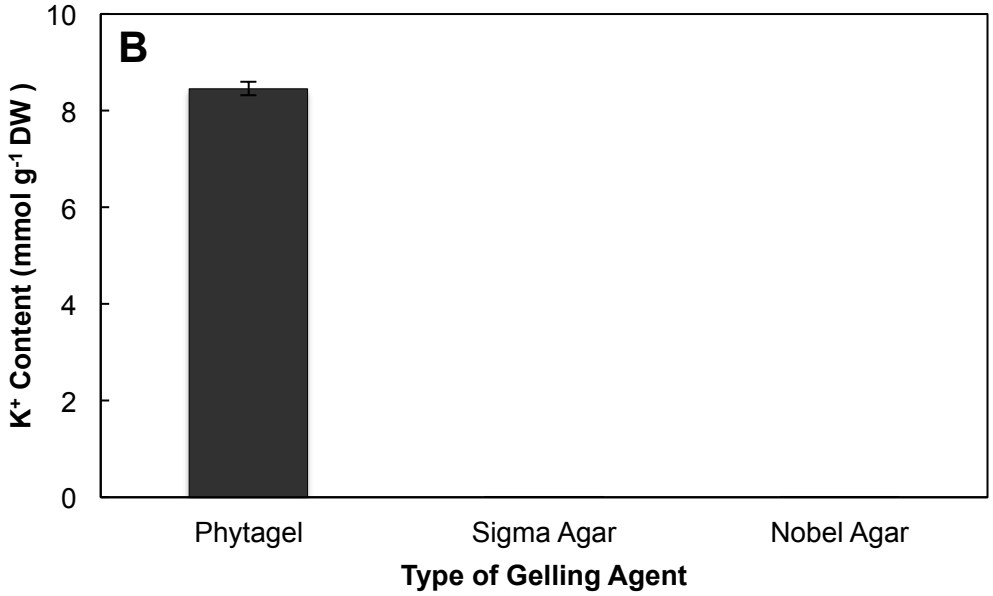

B
10
8
6
4
2
0
K+ Content (mmol g-1 DW)
Phytagel
Sigma Agar
Nobel Agar
Type of Gelling Agent

Figure 7 - K^+ content of 7-day-old *Arabidopsis* roots. Seedlings were grown on defined nutrient Phytagel plates prepared with varying K^+ content. Results show the mean of 4 biological replicates ± SE (n = 24 seedlings per species per treatment). Means of root K^+ contents were analyzed by a one-way ANOVA and were not significant between the 3 treatments ($P > 0.05$).

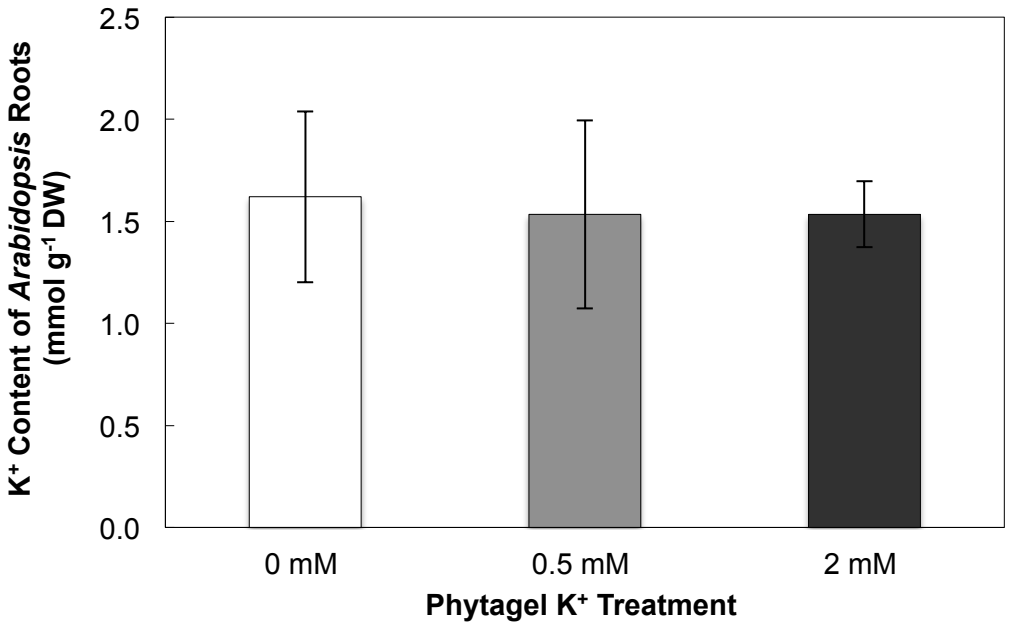
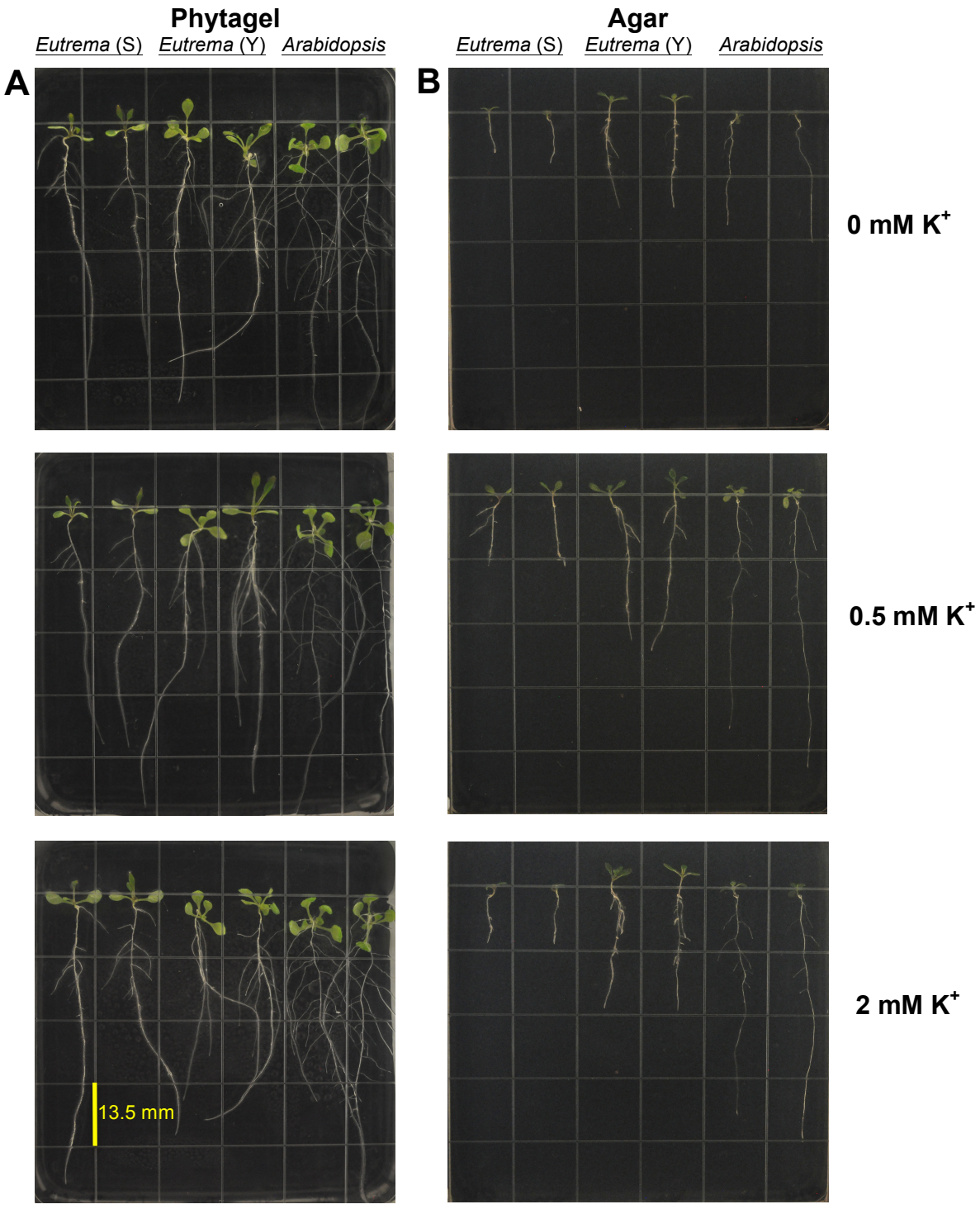
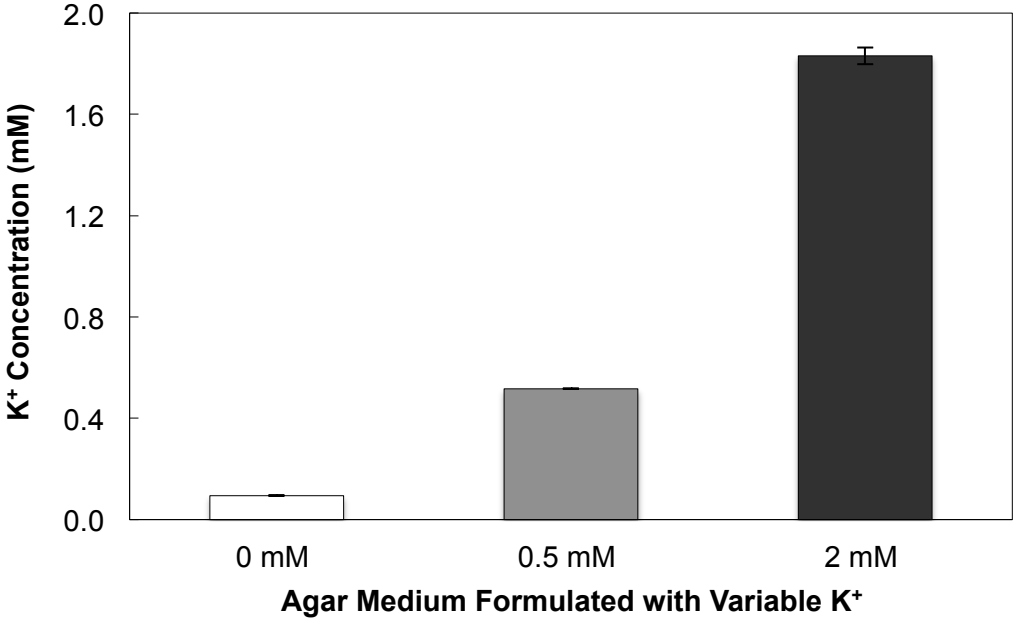

2.5
2.0
1.5
1.0
0.5
0.0
K+ Content of Arabidopsis Roots
(mmol g-1 DW)
0 mM
0.5 mM
2 mM
Phytagel K+ Treatment

Figure 8 - Representative 7-day-old Shandong *Eutrema* (*Eutrema* (S)), Yukon *Eutrema* (*Eutrema* (Y)), and *Arabidopsis* seedlings grown on a single nutrient plate solidified with (A) Phytagel or (B) Sigma agar (Cat. No. A1296, Batch No. SLBF1721V). Plates shown were exposed to identical conditions in the growth chamber (see Materials and Methods for light and temperature settings).


Phytagel
Eutrema (S)
Eutrema (Y)
Arabidopsis
A
Agar
Eutrema (S)
Eutrema (Y)
Arabidopsis
B
0 mM K+
0.5 mM K+
2 mM K+
13.5 mm

used as the solidifying agent instead of Phytagel. The seedlings grown on plates solidified with agar (Figure 8B) were smaller at the same stage of the experiment and the various plants were differentiated with respect to their response to the content of K^+. To confirm that the agar plates have less K^+ than the gels composed of Phytagel, we measured the K^+ content of the plates. As Figure 9 shows, nutrient plates solidified with agar contain significantly less K^+ than the Phytagel plates and the values for the K^+ concentrations are more in keeping with the K^+ that was added.

Figure 9 - K^+ content of Sigma agar plates used for root architecture and K^+ content experiments. Plates contained one of three K^+ concentrations with all other nutrients unchanged from those given in Table 1. K^+ was determined by atomic absorption spectrometry. For this analysis, an approximate 250 mg square plug was removed from the gel matrix of a defined nutrient plate and processed for K^+ content following the protocol outlined for tissue in the Materials and Methods section. Three conditions tested were: 0 mM K^+ (white), 0.5 mM K^+ (grey) and 2 mM K^+ (black). Sigma agar (Cat. No. A1296, Batch No. SLBF1721V) was used. Results show the means of 3 biological replicates ± SE. Data of nutrient plate K^+ content was analyzed by a one-way ANOVA and means were significant between the 3 treatments ($P < 0.05$).


2.0
1.6
1.2
0.8
0.4
0.0
K+ Concentration (mM)
0 mM
0.5 mM
2 mM
Agar Medium Formulated with Variable K+

II. Growth of seedlings on agar media formulated with variable K^+ content

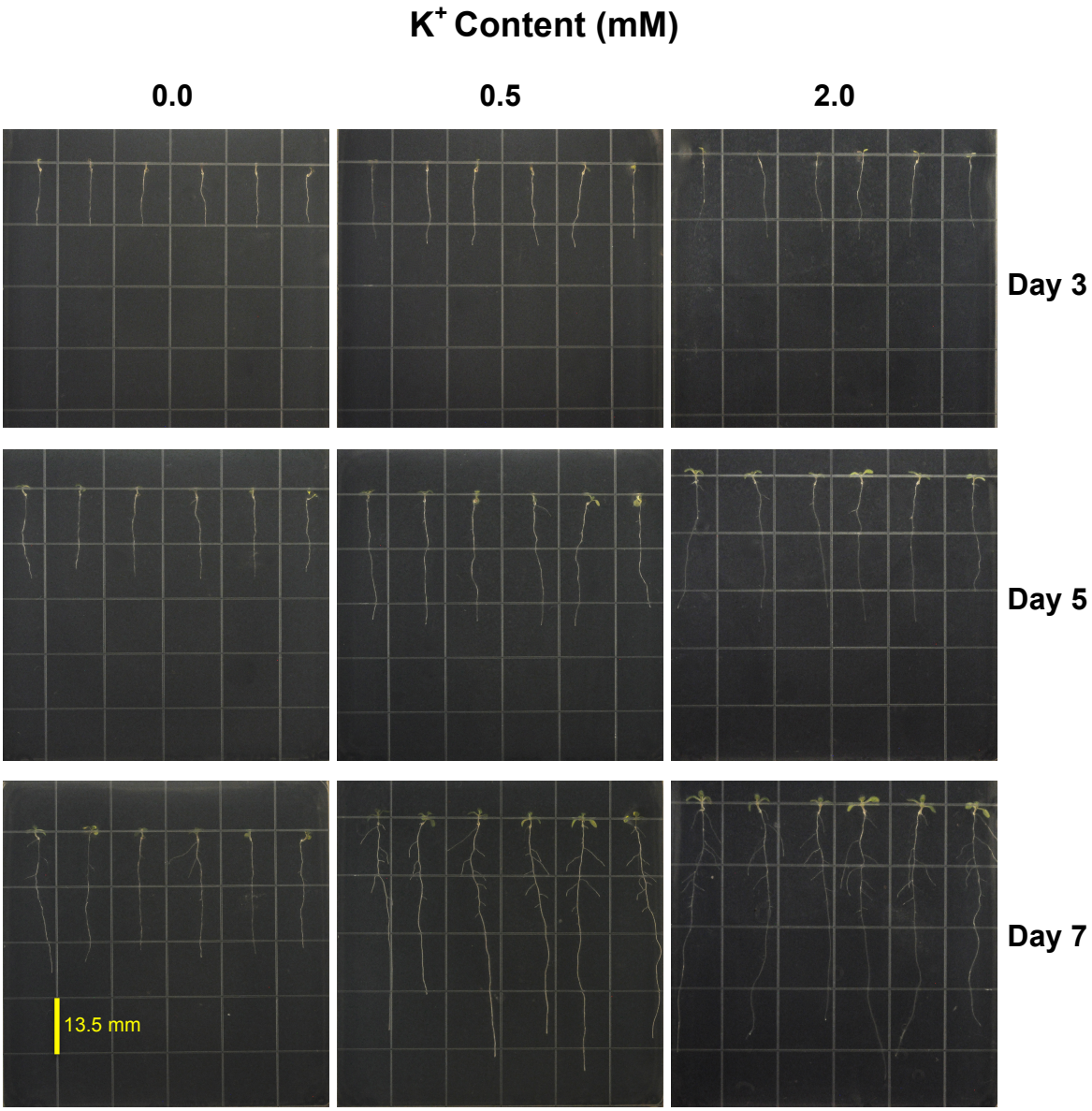
Arabidopsis seedlings undergo changes in root morphology, including the inhibition of lateral root growth, when responding to a deficiency in K^+ (Armengaud et al. 2004; Shin & Schachtman 2004; Kellermeier et al., 2013). Given the close relationship between *Eutrema* and this model species, we hypothesized that *Eutrema* seedlings would experience similar changes in root architecture when grown on K^+ deficient medium. Two *Eutrema* accessions were compared with respect to their growth on defined nutrient media with varying K^+: one native to the Yukon Territories of Canada and the other to the Shandong Province in China. To test this hypothesis, seeds of all three plants (*Arabidopsis,* Yukon and Shandong *Eutrema*) were germinated on MES medium and then transferred on the day of germination to nutrient plates prepared with varying concentrations of K^+ solidified with Sigma agar (A1296 - SLBF1721V).

In the following sections it is important to note that measurements concluded on different days in order to maximize the time available for root growth on the plates. Photos were taken daily allowing for daily measurements of the roots until the primary root reached the bottom of the plates when further root growth and continued data collection was no longer possible. *Arabidopsis* primary roots grew the fastest, nearing the bottom of the plates on Day 7 on 2 mM K^+ plates whereas *Eutrema* primary roots reached the same milestone on Day 10 for Yukon seedlings and Day 15 for Shandong seedlings.

a) Growth of Arabidopsis thaliana *seedlings on media of varying K^+ content*

Arabidopsis seedlings began to show visible signs of impaired root growth by Day 3 following transfer of the germinated seeds to 0 mM K^+ plates (Fig. 10). By Day 7,

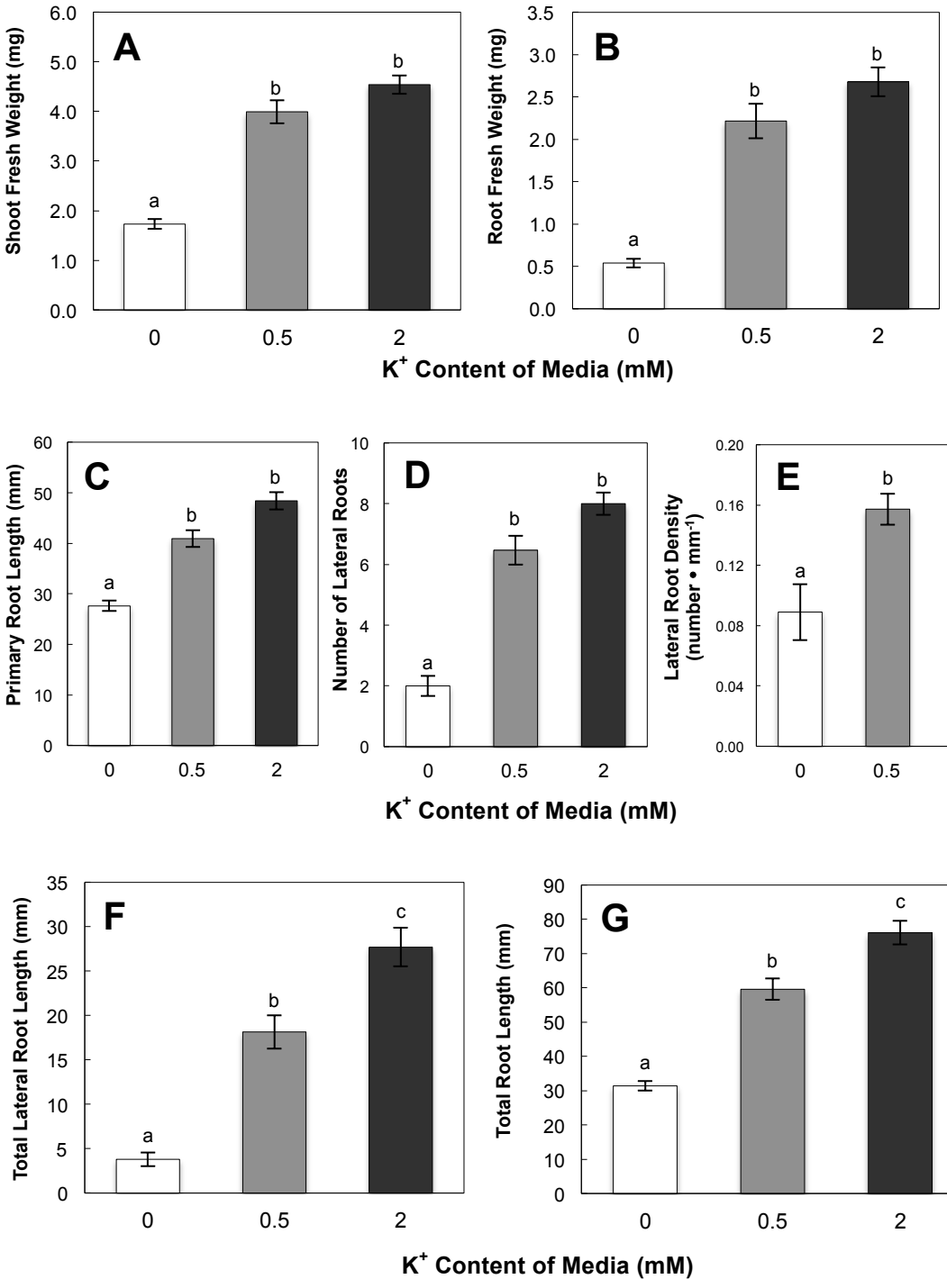
Figure 10 – *Arabidopsis thaliana* (Columbia ecotype) seedlings grown on defined nutrient media with variable K^+. Representative seedlings shown after 3, 5, and 7 d following transfer from MS germination plates to defined nutrient plates containing no added K^+ (0 mM K^+), 0.5 or 2 mM K^+. Seed germination and seedling growth conditions as outlined in the Materials and Methods section. The plates were prepared with Sigma agar (A1296 - SLBF1721V) and the details for preparation are given in the Materials and Methods with the composition reported in Table 1.


K+ Content (mM)
0.0
0.5
2.0
Day 3
Day 5
Day 7
13.5 mm

Arabidopsis seedlings on 0 mM K^+ media had visibly reduced shoot and root biomass compared to seedlings on 0.5 or 2 mM K^+ plates. This difference in growth was also seen in the quantitative measurements taken. The average shoot biomass for 2 mM K^+ seedlings was 4.54 ± 0.18 mg and while seedlings on 0.5 mM K^+ plates did not differ significantly in size, those seedlings on 0 mM K^+ had a fresh shoot biomass of 1.73 mg ± 0.10 mg thereby showing a 62% reduction in shoot biomass (Fig. 11A). Average root biomass for 2 mM K^+ seedlings was 2.68 ± 0.17 mg and again, seedlings grown on 0.5 mM K^+ plates did not significantly differ in fresh weight biomass (Fig. 11B). However, compared to the shoot biomass, root biomass was more negatively impacted by the lack of K^+ in that the seedling roots, at an average of 0.54 ± 0.05 mg, showed an 80% reduction in root biomass compared to the roots of seedlings grown on 2 mM K^+ (Fig. 11B).

Arabidopsis seedlings displayed significant reductions in the root growth parameters measured when seedlings on 0 mM K^+ plates were compared to those on 0.5 or 2 mM K^+ nutrient plates. In general, the reduction in root growth was less severe for seedlings on 0.5 mM versus 2.0 mM K^+ media. For example, with respect to primary root length, seedlings on 0 mM and 0.5 mM K^+ were 43% and 16% shorter, respectively, than the primary roots of seedlings on 2 mM K^+ plates (Fig. 11C). Seedling lateral root number, lateral root density, total lateral root length and total root length were also adversely affected by a lack of added K^+ in the medium relative to 2 mM K^+ (Fig. 11D to G). Seedlings on 0 mM K^+ plates showed a 75% reduction in the number of lateral roots compared to 2 mM K^+ media and for the same comparison, the total lateral root length

Figure 11 – Day 7 biomass and root measurements for *Arabidopsis thaliana* seedlings on defined nutrient media containing variable K^+. Measurements were taken 7 d following transfer of germinated seeds from MS plates to defined nutrient plates containing no added K^+ (0 mM K^+), 0.5 or 2 mM K^+. Panels show data for: (A) Shoot and (B) Root biomass, (C) Primary root length, (D) Number of lateral roots, (E) Lateral root density, (F) Total lateral root length and (G) Total root length. The plates were prepared with Sigma agar (A1296 - SLBF1721V) and the details for preparation are given in the Materials and Methods with the composition reported in Table 1. Data shown are the mean ± SE of 2 biological replicates (n = 45 seedlings). A one-way ANOVA was used to test for significance between treatments. For each panel, the bars designated by the same letter are not significantly different ($P > 0.05$).


A
Shoot Fresh Weight (mg)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a
b
b
0
0.5
2
B
Root Fresh Weight (mg)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
0
0.5
2
K+ Content of Media (mM)
C
Primary Root Length (mm)
60
50
40
30
20
10
0
a
b
b
0
0.5
2
D
Number of Lateral Roots
10
8
6
4
2
0
a
b
b
0
0.5
2
E
Lateral Root Density (number • mm-1)
0.20
0.16
0.12
0.08
0.04
0.00
a
b
b
0
0.5
2
K+ Content of Media (mM)
F
Total Lateral Root Length (mm)
35
30
25
20
15
10
5
0
a
b
c
0
0.5
2
G
Total Root Length (mm)
90
80
70
60
50
40
30
20
10
0
a
b
c
0
0.5
2
K+ Content of Media (mM)

also decreased by approximately 86%. With shorter primary roots and fewer lateral roots, a significant 44 and 48% reduction in lateral root density was not surprising for roots of seedlings on 0 mM K^+ relative to 0.5 and 2 mM K^+ plates, respectively. The total root lengths were calculated by taking the sum of the total lateral and primary root length for each plant and the mean value for 45 seedlings is reported in Figure 11G. On average, seedlings showed an almost 60% reduction in total root length on 0 mM K^+ media compared to seedlings on 2 mM K^+ plates. A comparison of Fig. 11C and 11F indicates that a major source in lost total root length came about by a reduction in lateral root development as opposed to primary root length.

b) Growth of Yukon Eutrema salsugineum *seedlings on media of varying K^+ content*

Similar to *Arabidopsis*, Yukon *Eutrema* seedlings showed visible signs of growth impairment on media lacking added K^+ (0 mM K^+) and this impact was seen within 5 d of seedling transfer from MS to K^+ treatment plates (Fig. 12). On Day 10, the final day of the experiment, the seedlings had significantly reduced shoot and root biomass on 0 mM K^+ media relative to media containing added K^+ (Fig. 12, 13A and B). Seedlings on 2 mM K^+ plates had an average shoot fresh weight of 4.05 ± 0.19 mg and those on 0 K^+ mM plates averaged 2.71 ± 0.09 mg indicating the seedlings on 0 mM K^+ had 33% less shoot biomass compared to seedlings on 2 mM K^+ medium (Fig. 13A). In terms of root biomass, Yukon *Eutrema* seedlings grown on 2 mM K^+ plates had an average root fresh weight of 2.94 ± 0.18 mg whereas seedlings grown on 0 mM K^+ plates had an average root fresh weight 1.31 ± 0.05 mg thereby showing a 55% reduction in root biomass

Figure 12 - Yukon *Eutrema salsugineum* seedlings grown on defined nutrient media with variable K^+. Representative seedlings shown after 5, 7, and 10 d following transfer of germination seeds from MS plates to defined nutrient plates containing no added K^+ (0 mM K^+), 0.5 or 2 mM K^+. Seed germination and seedling growth conditions as outlined in the Materials and Methods section. The plates were prepared with Sigma agar (A1296 - SLBF1721V) and the details for preparation are given in the Materials and Methods with the composition reported in Table 1.

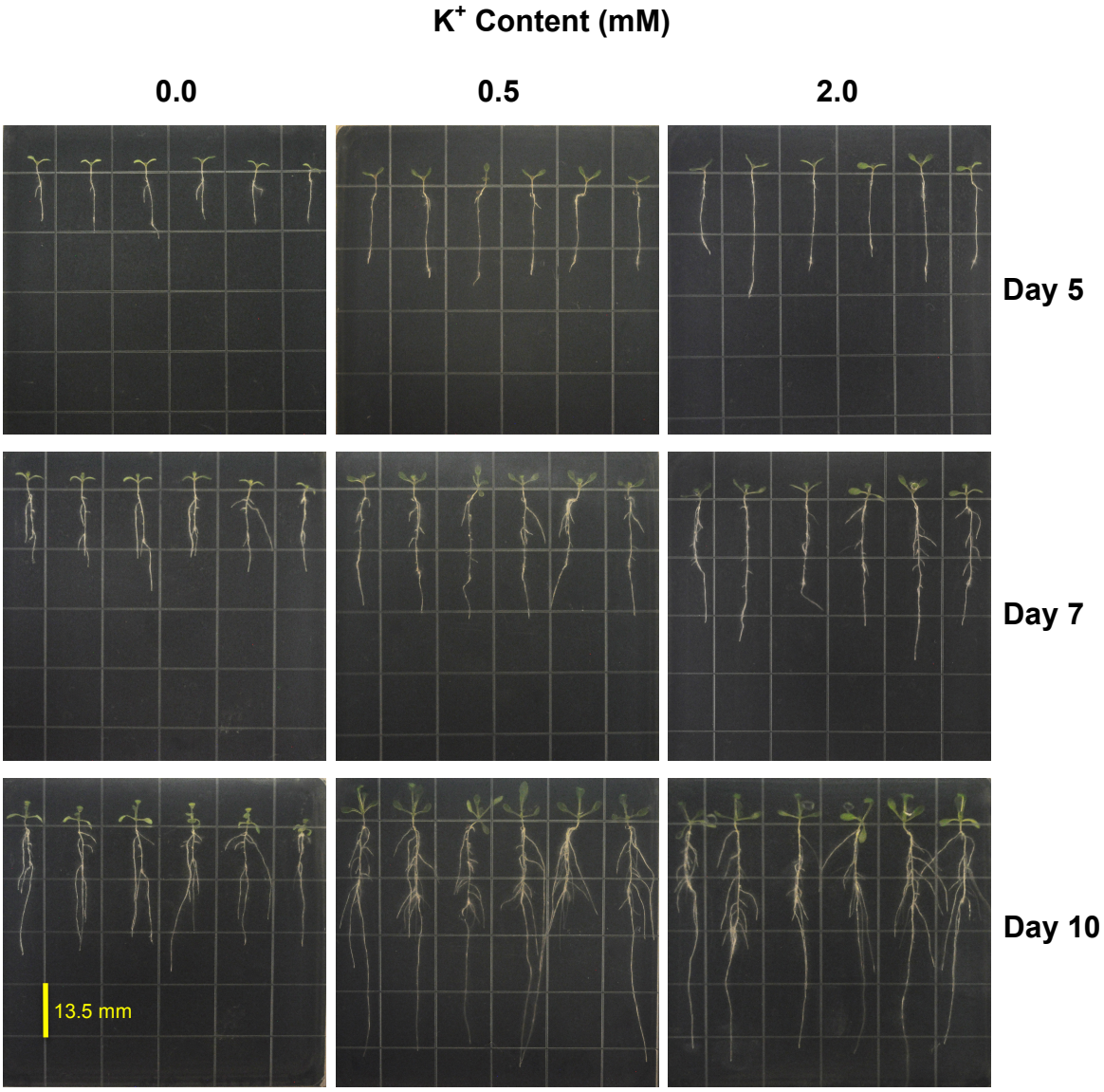
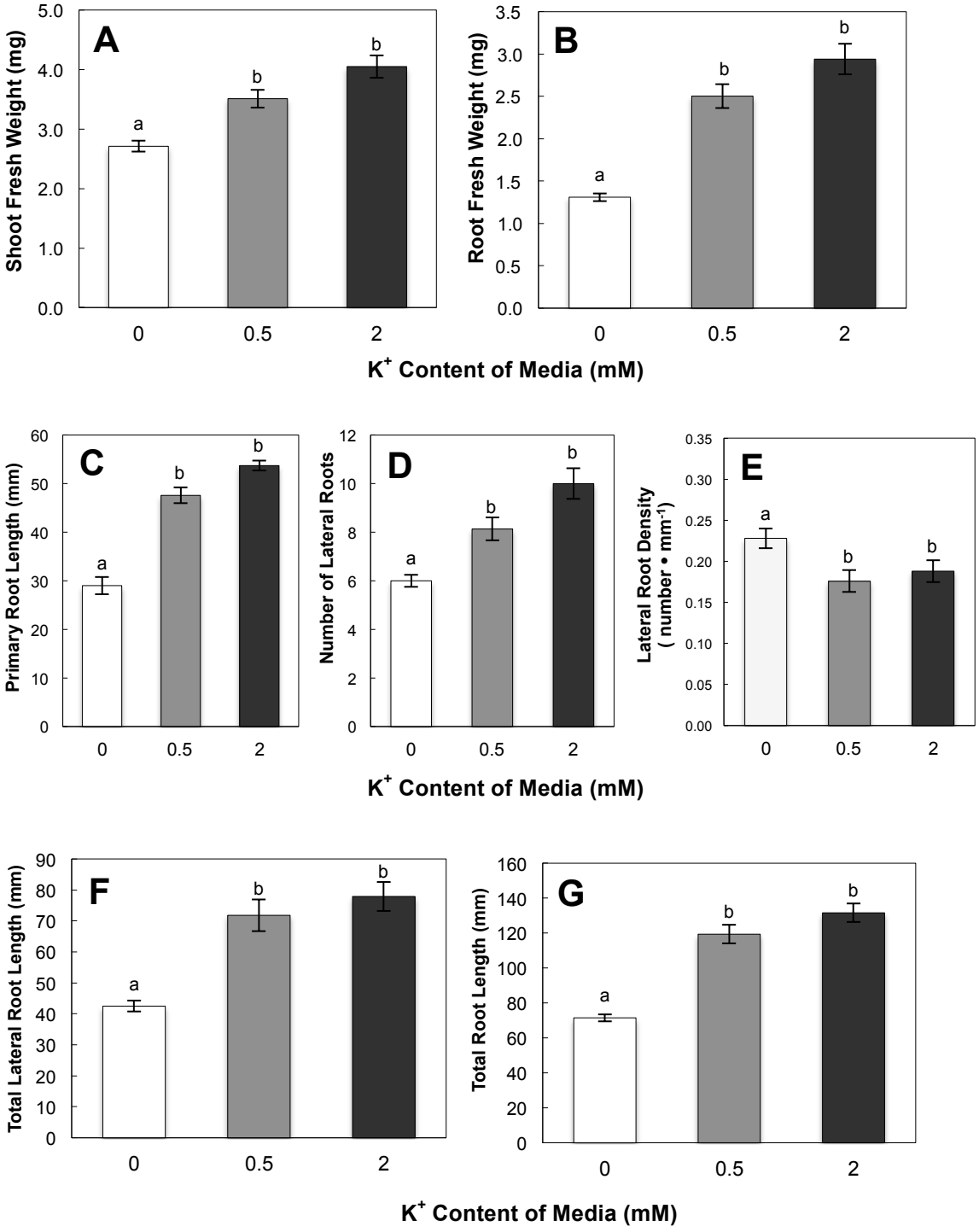

K+ Content (mM)
0.0
0.5
2.0
Day 5
Day 7
Day 10
13.5 mm

Figure 13 – Day 10 biomass and root measurements for Yukon *Eutrema salsugineum* seedlings on defined nutrient media containing variable K^+. Measurements were taken 10 d following transfer of germinated seeds from MS plates to defined nutrient plates containing no added K^+ (0 mM K^+), 0.5 or 2 mM K^+. Panels show data for: (A) Shoot and (B) Root biomass, (C) Primary root length, (D) Number of lateral roots, (E) Lateral root density, (F) Total lateral root length and (G) Total root length. The plates were prepared with Sigma agar (A1296 - SLBF1721V) and the details for preparation are given in the Materials and Methods with the composition reported in Table 1. Data shown are the mean ± SE of 2 biological replicates (n = 45 seedlings). A one-way ANOVA was used to test for significance between the treatments. For each panel, the bars designated by the same letter are not significantly different ($P > 0.05$).


A
Shoot Fresh Weight (mg)
5.0
4.0
3.0
2.0
1.0
0.0
a
b
b
0
0.5
2
B
Root Fresh Weight (mg)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
0
0.5
2
K+ Content of Media (mM)
C
Primary Root Length (mm)
60
50
40
30
20
10
0
a
b
b
0
0.5
2
D
Number of Lateral Roots
12
10
8
6
4
2
0
a
b
b
0
0.5
2
E
Lateral Root Density
(number • mm-1)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
b
b
0
0.5
2
K+ Content of Media (mM)
F
Total Lateral Root Length (mm)
90
80
70
60
50
40
30
20
10
0
a
b
b
0
0.5
2
G
Total Root Length (mm)
160
140
120
100
80
60
40
20
0
a
b
b
0
0.5
2
K+ Content of Media (mM)

relative to the seedlings on 2 mM K^+ plates (Fig. 13B). Thus the growth of shoots was less adversely impacted by the lack of K^+ in the medium than was the growth of roots.

Several features of the roots were negatively impacted by the absence of K^+ from the medium formulation. Primary roots of Yukon *Eutrema* seedlings growing on 0 mM K^+ plates were 46% shorter, the number of lateral roots was reduced by 40%, total lateral root length was reduced by 46% and total root length by 46% relative to roots of seedlings on 2 mM K^+ plates (Fig. 13C,D, F and G). Relative to the results for *Arabidopsis,* the results for lateral root densities at variable K^+ exposure were different for Yukon *Eutrema.* Instead of showing a decrease in lateral root density on 0 mM K^+ plates as was the case for *Arabidopsis* (Fig. 11E), Yukon *Eutrema* plants had a 21% higher lateral root density relative to the lateral root density of seedlings on 2 mM K^+ plates. This indicates that the number of lateral roots per unit length of primary root changed but not to the extent or direction as seen for *Arabidopsis* seedlings exposed to the same K^+ treatment conditions.

c) Growth of Shandong Eutrema salsugineum *seedlings on media of varying K^+ content*

Shandong *Eutrema* seedlings showed signs of a K^+ deficiency within 7 d of the experiment and by the final day of experimentation, seedlings grown on 0 mM K^+ medium had a visibly and measurably reduced shoot and root biomass compared to seedlings grown on 0.5 mM or 2 mM K^+ plates (Fig. 14, Fig. 15A and B). Although it took an additional 5 d, the average shoot fresh weight for Shandong seedlings grown on 2 mM K^+ plates at 5.16 ± 0.47 mg and root biomass at 1.63 ± 0.19 mg had just exceeded the average shoot biomass but not the root biomass values obtained by Yukon seedlings at

Figure 14 – Shandong *Eutrema salsugineum* seedlings grown on defined nutrient media with variable K^+. Representative seedlings shown after 7, 10, and 15 d following transfer of germinated seeds from MS plates to defined nutrient plates containing no added K^+ (0 mM K^+), 0.5 or 2 mM K^+. Seed germination and seedling growth conditions as outlined in the Materials and Methods section. The plates were prepared with Sigma agar (A1296 - SLBF1721V) and the details for preparation are given in the Materials and Methods with the composition reported in Table 1.

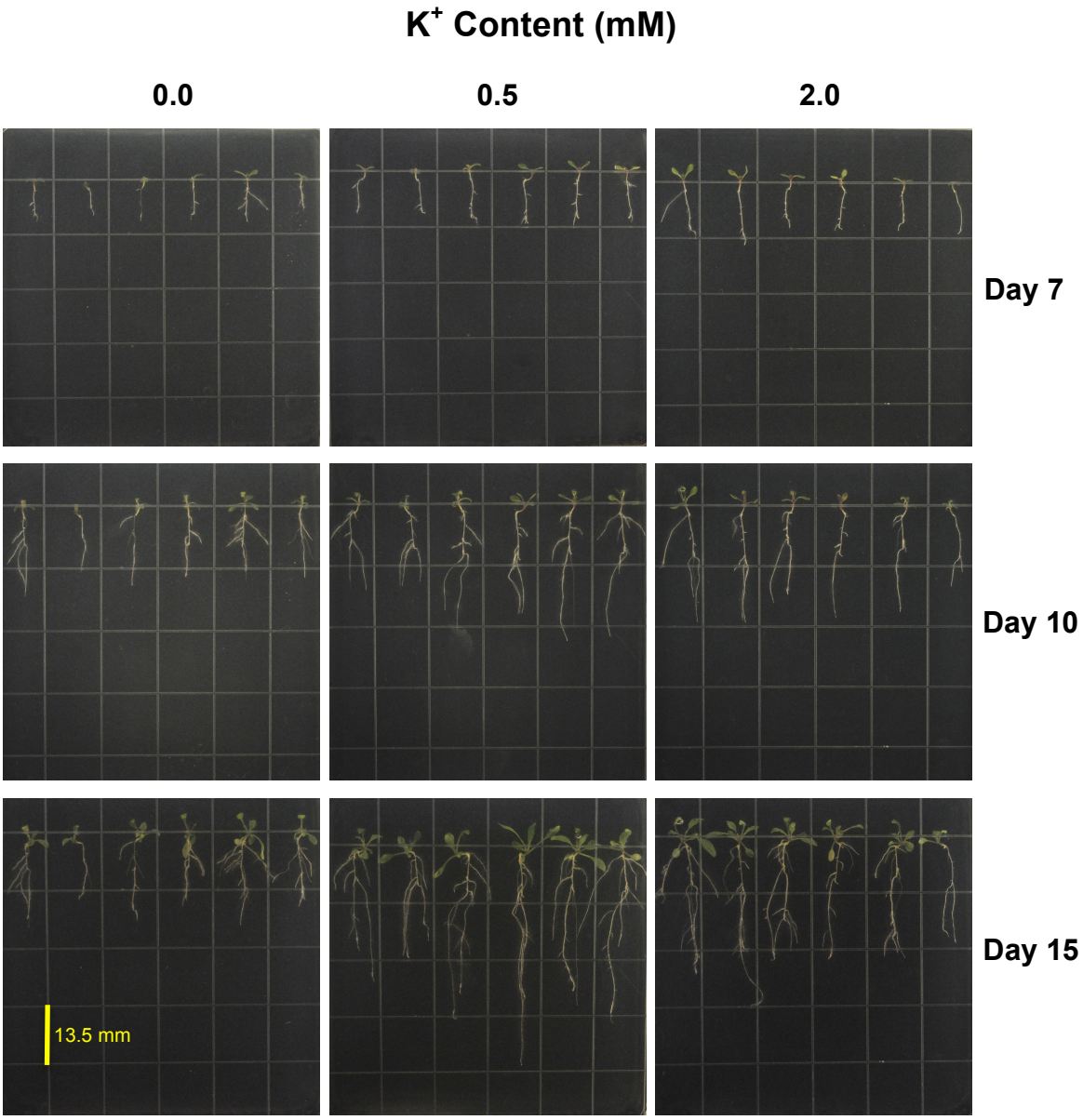
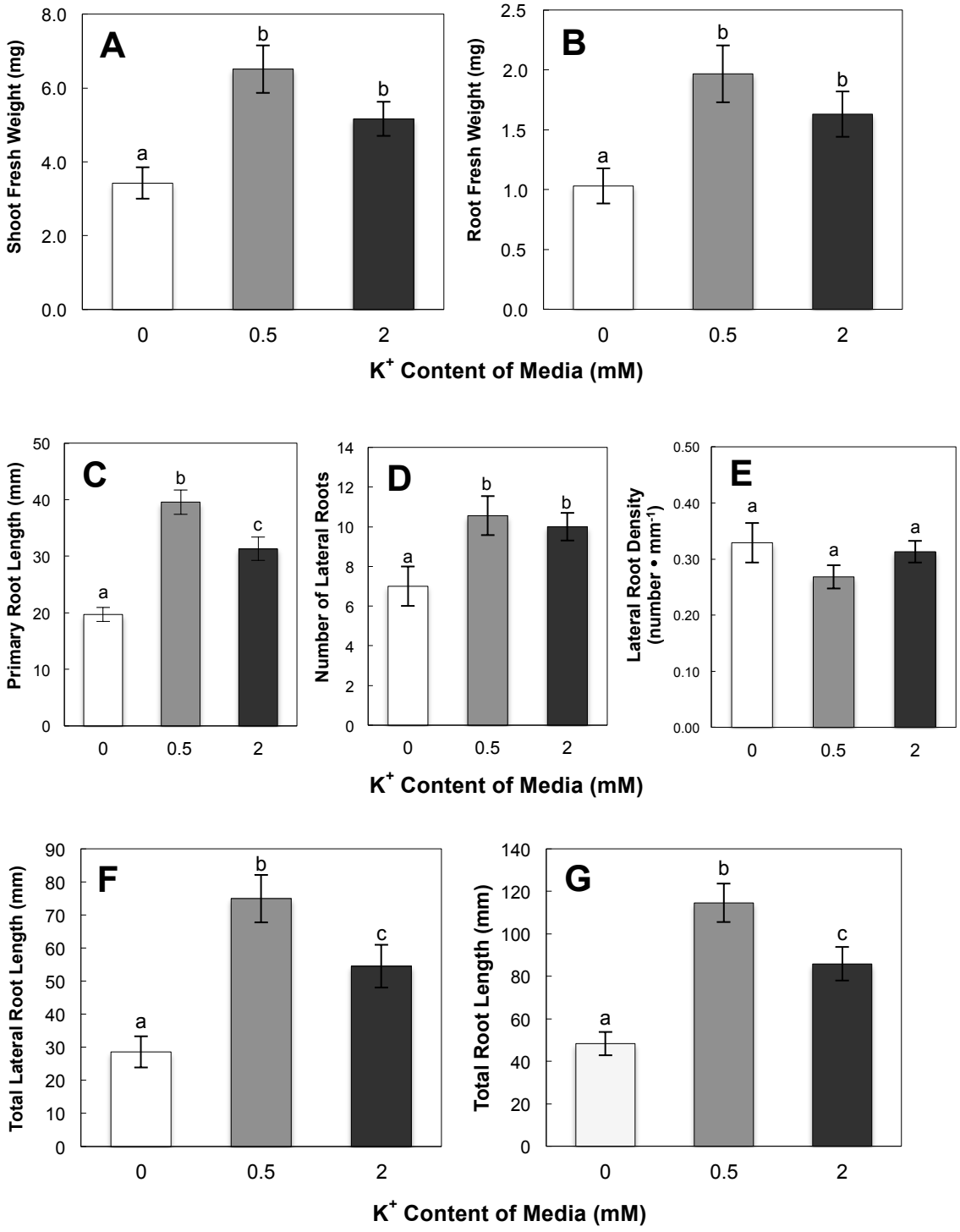

K+ Content (mM)
0.0
0.5
2.0
Day 7
Day 10
Day 15
13.5 mm

Figure 15 - Day 15 biomass and root measurements for Shandong *Eutrema salsugineum* seedlings on defined nutrient media containing variable K^+. Measurements were taken 15 d following transfer of germinated seeds from MS plates to defined nutrient plates containing no added K^+ (0 mM K^+), 0.5 or 2 mM K^+. Panels show data for: (A) Shoot and (B) Root biomass, (C) Primary root length, (D) Number of lateral roots, (E) Lateral root density, (F) Total lateral root length and (G) Total root length. The plates were prepared with Sigma agar (A1296 - SLBF1721V) and the details for preparation are given in the Materials and Methods with the composition reported in Table 1. Data shown are the mean ± SE of 2 biological replicates (n = 36 seedlings). A one-way ANOVA was used to test for significance between the treatments. For each panel, the bars designated by the same letter are not significantly different ($P > 0.05$).


A
Shoot Fresh Weight (mg)
8.0
6.0
4.0
2.0
0.0
a
b
b
0
0.5
2
B
Root Fresh Weight (mg)
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
0
0.5
2
K+ Content of Media (mM)
C
Primary Root Length (mm)
50
40
30
20
10
0
a
b
c
0
0.5
2
D
Number of Lateral Roots
14
12
10
8
6
4
2
0
a
b
b
0
0.5
2
E
Lateral Root Density (number • mm-1)
0.50
0.40
0.30
0.20
0.10
0.00
a
a
a
0
0.5
2
K+ Content of Media (mM)
F
Total Lateral Root Length (mm)
90
80
70
60
50
40
30
20
10
0
a
b
c
0
0.5
2
G
Total Root Length (mm)
140
120
100
80
60
40
20
0
a
b
c
0
0.5
2
K+ Content of Media (mM)

Day 10 (values were 4.05 ± 0.19 mg and 2.94 ± 0.18 mg, for Yukon shoot and root biomass, respectively). The reduction in both shoot and root biomass between Shandong seedlings grown on 0 mM K^+ plates versus 0.5 and 2 mM K^+ plates was about 48% (Fig. 15A, B). However, unlike Yukon *Eutrema* or *Arabidopsis*, Shandong *Eutrema* seedling roots and shoots grew as well, if not somewhat better, on the 0.5 mM K^+ compared to 2 mM K^+ plates (Fig. 14). This difference in performance was seen in the measurements of the roots that were determined in that the primary root, lateral root, and total root lengths were all significantly higher by approximately 25% for seedlings on 0.5 mM K^+ relative to 2 mM K^+ plates (Fig. 15C, F, G). When comparing seedlings growing on 0 mM K^+ plates, primary roots of Shandong *Eutrema* seedlings were 50% shorter, total lateral root length was reduced by 62% and total root length by 58% relative to roots of seedlings on 0.5 mM K^+ plates (Fig. 15C, F and G). Another difference between the three plants tested is that while seedlings on 0 mM K^+ plates had fewer lateral roots compared to seedlings on 0.5 or 2 mM K^+ plates, there was no significant treatment-related difference in lateral root density (Fig. 15D and E). This suggests that any change in primary root length with altered K^+ content in the medium was accompanied by a corresponding change in the number of lateral roots resulting in no net change in lateral root density, an impression reinforced by the appearance of the seedlings shown in Figure 14.

III. K^+ content measurements for roots and shoots of seedlings grown on defined nutrient agar

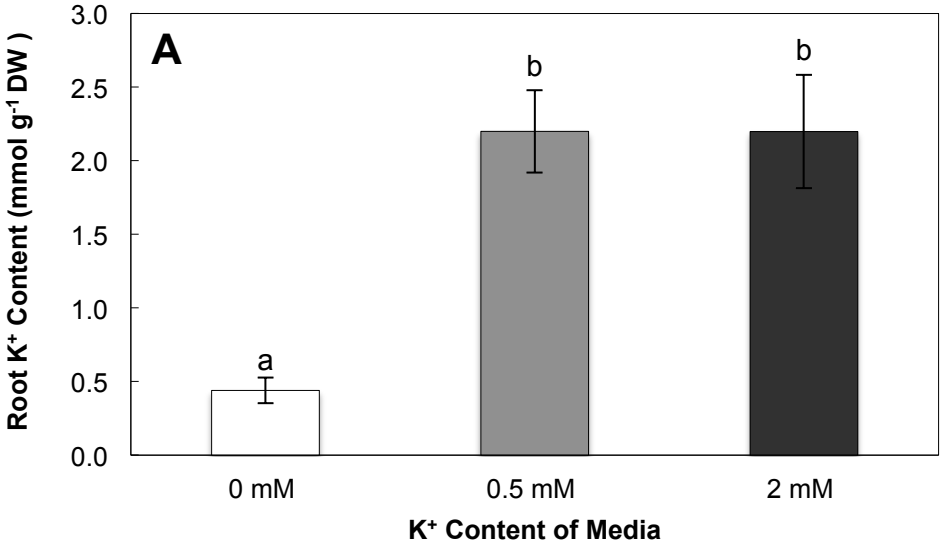
Seedling root and shoot K^+ content measurements were taken for each of the plants on the final day of experimentation. On Day 10, the only statistically significant

difference in K^+ content for *Arabidopsis* roots and shoots was found for the seedlings on plates where no additional K^+ was added (0 mM K^+; Fig. 16). A similar outcome was observed for *Eutrema* seedlings. Figures 17 and 18 show the seedling root and shoot K^+ content data for 10-day-old Yukon and 15-day-old Shandong *Eutrema* seedlings, respectively. The 0 mM K^+ plates consistently yielded *Eutrema* seedlings with the lowest root and shoot K^+ content. This finding shows that the seedlings respond to the K^+ content of the treatment plates with the seedlings showing slower growth (Fig. 8B) and lowest K^+ content on the plates with the least amount of K^+ present (Fig. 9).

a) Root K^+ Content

Arabidopsis and Yukon *Eutrema* seedlings grown on the agar plates with the three K^+ contents responded similarly with respect to average K^+ content per g DW of tissue (Figs. 16 and 17). Both roots showed a similar reduction in K^+ content, on the order of about 79 to 80%, between seedlings on the 0 mM and the 0.5 or 2.0 mM K^+ treatment plates. Shandong *Eutrema* seedlings, at approximately 1.2 mmol g^{-1} DW, had a lower root K^+ content on 0.5 mM and 2.0 mM K^+ plates relative to Yukon *Eutrema* or *Arabidopsis* seedlings (Fig. 18) and interestingly, the decrease in K^+ content per g DW experienced by Shandong seedlings on the 0 mM medium relative to 0.5 or 2.0 mM K^+ at 84% was also close to what was found by way of reduced K^+ content for Yukon *Eutrema* and *Arabidopsis* roots.

Figure 16 - K^+ content of *Arabidopsis* seedlings grown on defined nutrient agar plates of varying K^+ content. (A) Roots and (B) Shoots were harvested from seedlings grown for 10 d on 0 mM K^+ (white), 0.5 mM K^+ (grey) or 2 mM K^+ (black) treatment plates (see Materials and Methods for plate formulation details). Atomic absorption was used to determine the K^+ content of the tissues and the data is reported as a function of tissue DW. Data shown are the means ± SE of 3 biological replicates (n = 18 seedlings per species per treatment). A one-way ANOVA was used to test for significance between treatments. For each panel, the bars designated by the same letter are not significantly different ($P > 0.05$).


A
Root K+ Content (mmol g-1 DW)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
0 mM
0.5 mM
2 mM
K+ Content of Media

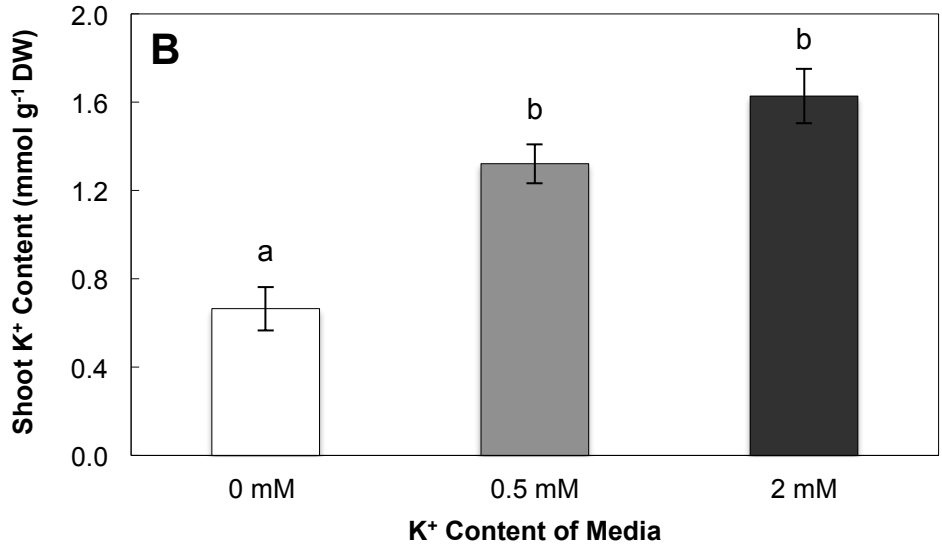
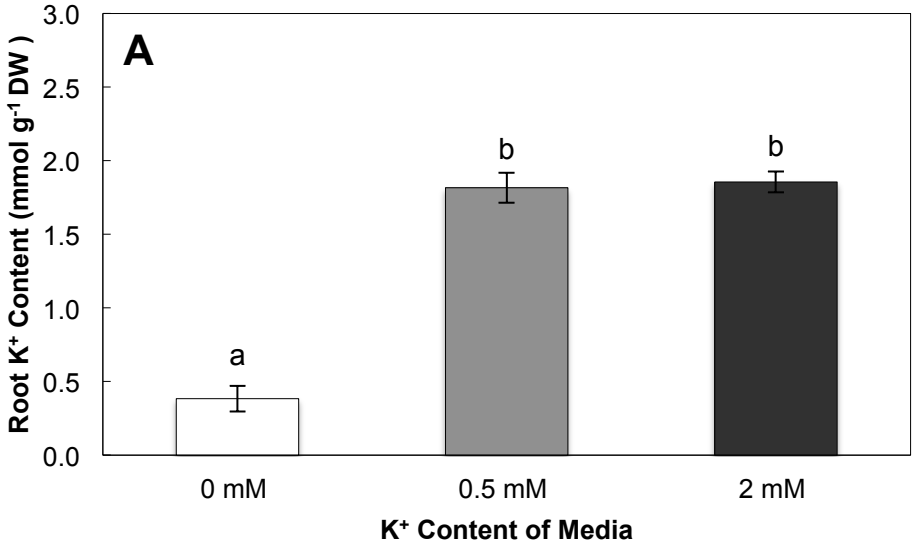

B
Shoot K+ Content (mmol g-1 DW)
2.0
1.6
1.2
0.8
0.4
0.0
a
b
b
0 mM
0.5 mM
2 mM
K+ Content of Media

Figure 17 - K^+ content of Yukon *Eutrema* seedlings grown on defined nutrient agar plates of varying K^+ content. (A) Roots and (B) Shoots were harvested from seedlings grown for 10 d on 0 mM K^+ (white), 0.5 mM K^+ (grey) or 2 mM K^+ (black) treatment plates (see Materials and Methods for plate formulation details). Atomic absorption was used to determine the K^+ content of the tissues and the data is reported as a function of tissue DW. Data shown are the means ± SE of 3 biological replicates (n = 18 seedlings per species per treatment). A one-way ANOVA was used to test for significance between treatments. For each panel, the bars designated by the same letter are not significantly different ($P > 0.05$).


A
Root K+ Content (mmol g-1 DW)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
0 mM
0.5 mM
2 mM
K+ Content of Media

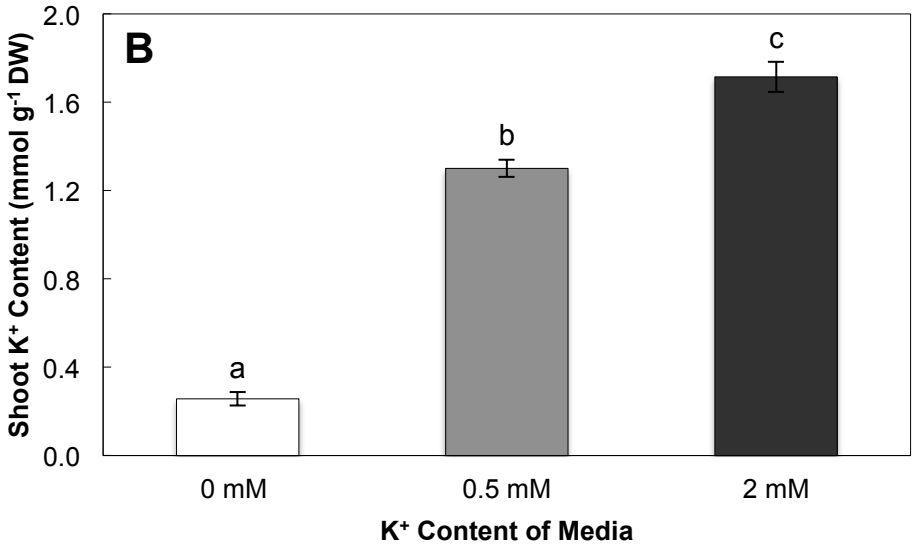
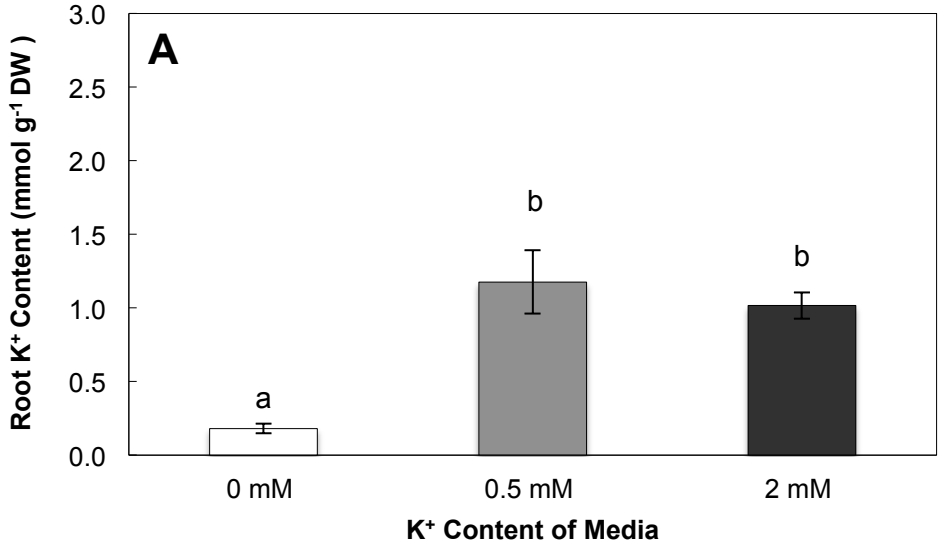
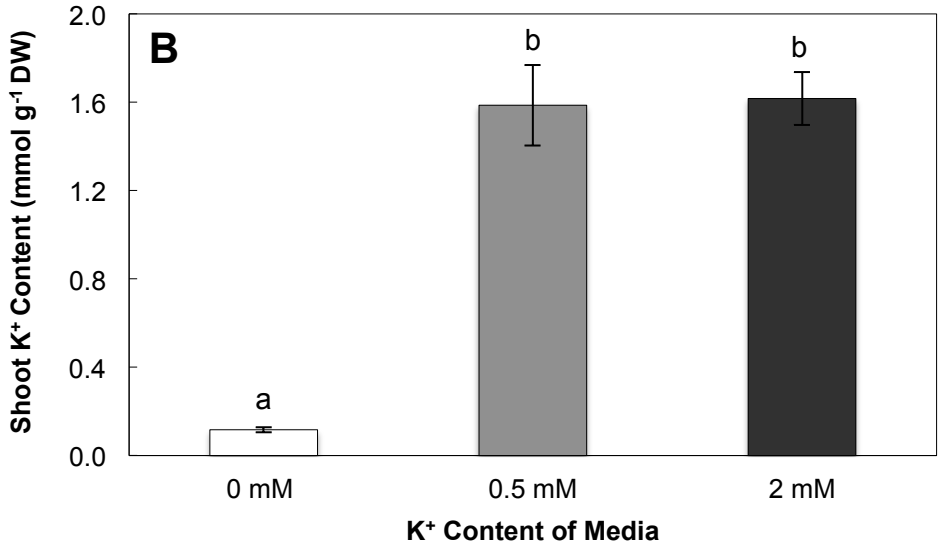

B
Shoot K+ Content (mmol g-1 DW)
2.0
1.6
1.2
0.8
0.4
0.0
a
b
c
0 mM
0.5 mM
2 mM
K+ Content of Media

Figure 18 - K^+ content of Shandong *Eutrema* seedlings grown on defined nutrient agar plates of varying K^+ content. (A) Roots and (B) Shoots were harvested from seedlings grown for 15 d on 0 mM K^+ (white), 0.5 mM K^+ (grey) or 2 mM K^+ (black) treatment plates (see Materials and Methods for plate formulation details). Atomic absorption was used to determine the K^+ content of the tissues and the data is reported as a function of tissue DW. Data shown are the means ± SE of 3 biological replicates (n = 18 seedlings per species per treatment). A one-way ANOVA was used to test for significance between treatments. For each panel, the bars designated by the same letter are not significantly different ($P > 0.05$).


A
Root K+ Content (mmol g-1 DW)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
0 mM
0.5 mM
2 mM
K+ Content of Media


B
Shoot K+ Content (mmol g-1 DW)
2.0
1.6
1.2
0.8
0.4
0.0
a
b
b
0 mM
0.5 mM
2 mM
K+ Content of Media

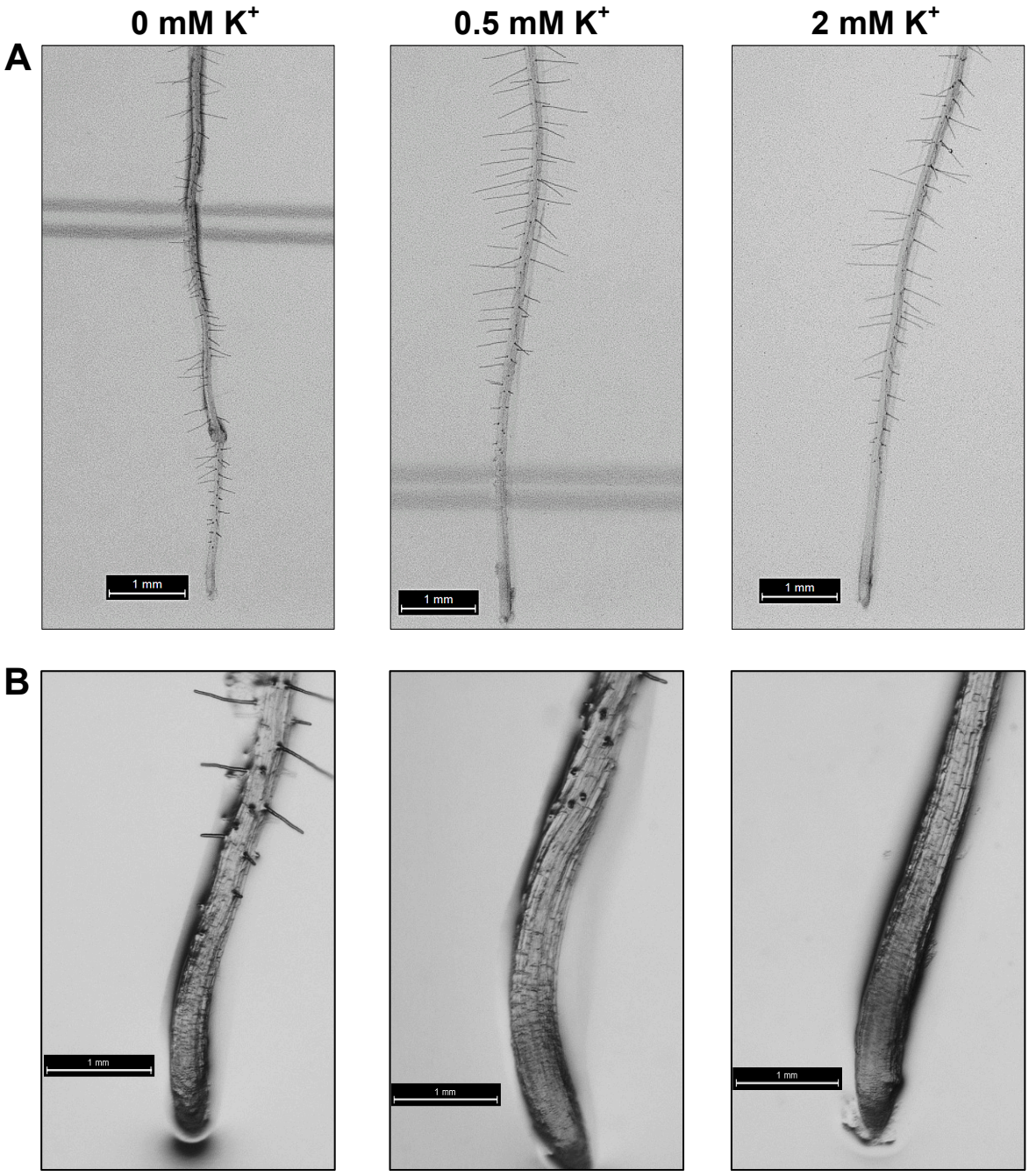
b) Shoot K^+ Content

In general, shoot K^+ content showed less variability between *Arabidopsis,* Yukon and Shandong *Eutrema* seedlings grown on 0.5 mM K^+ or 2.0 mM K^+ treatment plates and values ranged from about 1.3 to 1.6 mmol g^{-1} DW. As with the roots, the K^+ content of the shoots was lowest on the agar without K^+ added (0 mM K^+) but the extent of the reduction between 0 mM and 2 mM K^+ treatment plates was variable between the plants tested. The greatest reduction in K^+ content per g DW was found for Shandong *Eutrema* shoots at approximately 93% between seedlings grown for 15 d on 2 mM K^+ versus 0 mM K^+ agar (Fig. 18). For the same comparison, the least change in shoot K^+ content was found for *Arabidopsis* seedlings at 59% while Yukon *Eutrema* shoots underwent the same relative reduction in K^+ content as their roots at about 84% (Figs. 16 and 17).

IV. Seedling root hairs show treatment-responsive variability

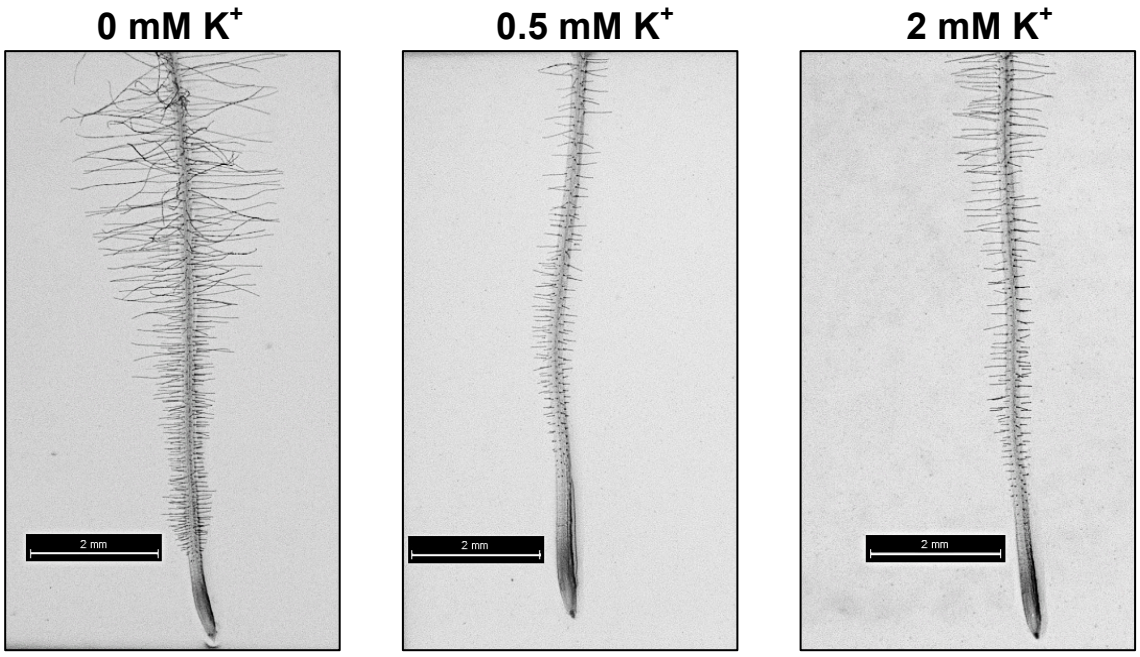
Root hairs are the extensions of single epidermal cells and they can significantly increase the absorptive surface area for roots to water and nutrient uptake (Desbrosses et al., 2003). In addition to the differences in root architecture between the three plants used, there were also indications that root hair development was different depending on the plant and the K^+ content of the medium. Without the benefit of a microscope, the root hairs of *Arabidopsis* seedlings grown on the various K^+ treatments did not look very different. Figure 19-A shows pictures of the bottom of primary roots examined under the microscope and it was still difficult to see if seedlings showed any physical changes in root hair growth on K^+ deficient media. However, Figure 19-B shows the apical region of the primary roots of *Arabidopsis* seedlings grown on 0, 0.5 and 2.0 mM K^+. Root hairs

Figure 19 - Root hairs of *Arabidopsis* seedlings grown on defined nutrient media of varying K^+ concentrations. Representative four-day-old *Arabidopsis* seedlings grown in the presence of 0 mM, 0.5 mM and 2 mM K^+. Pictures show (A) apical region of the bottom of primary roots and (B) a magnified tip of a primary root to show the relative emergence of root hairs near the apex. The magnification scales are shown.


0 mM K+
0.5 mM K+
2 mM K+
A
B
1 mm
1 mm
1 mm
1 mm
1 mm
1 mm

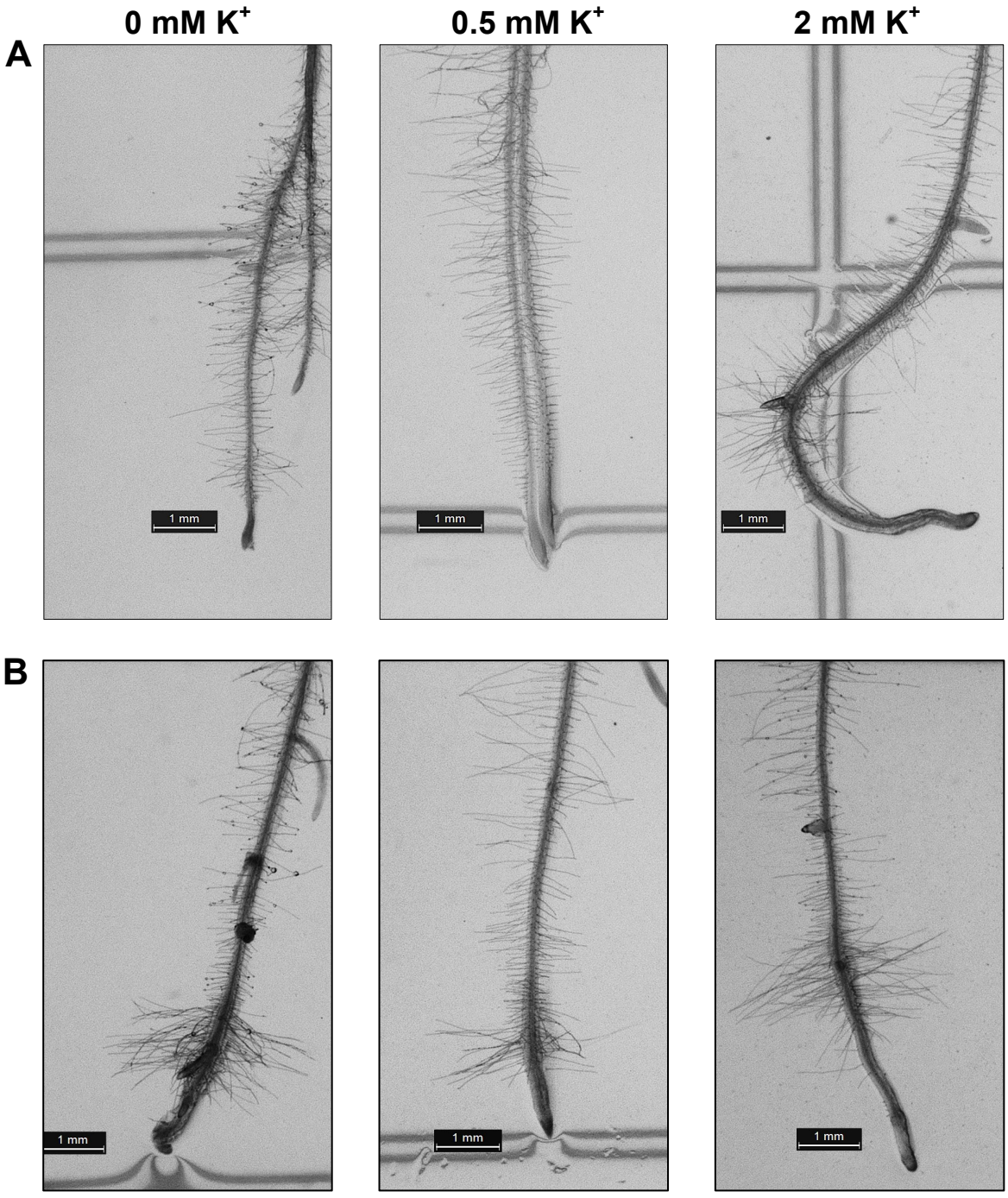
are formed closer to the tip of the primary root of 4-day-old *Arabidopsis* seedlings with decreasing K^+ content in the medium. Figure 20 shows that root hair growth at the base of primary roots of 10-day-old Yukon *Eutrema* seedlings were also closest to the apical root tip when grown on 0 mM K^+ plates but the root hairs were also consistently longer than those of seedlings grown in the presence of 0.5 mM or 2 mM K^+. Preliminary measurements of these root hairs were taken and they showed that 0 mM K^+ grown Yukon *Eutrema* seedlings have root hairs that are on average 1 mm longer than those of seedlings grown in the presence of added K^+, which is significant given that root hairs are the extension of a single epidermal cell and this change represents a 2.75-fold increase in length.

Figure 20 - Root hairs of Yukon *Eutrema* seedlings grown on defined nutrient media of varying K^+ concentrations. Representative 10-day-old *Eutrema* seedlings grown in the presence of 0 mM, 0.5 mM and 2 mM K^+. Pictures show the magnified tip of a primary root and the magnification scale is given.


0 mM K+
0.5 mM K+
2 mM K+
2 mm
2 mm
2 mm

Shandong seedlings, on the other hand, had similar root hair growth patterns on media of varying K^+ content (Fig. 21A). However, about 50 to 60% of the seedlings showed a proliferation of lengthy root hairs by Day 15 that were in an area near the root tip that was flanked by zones where the root hairs were shorter giving an appearance of sporadic "tufts" of hairs (Fig. 21B).

Figure 21 - Root hairs of Shandong *Eutrema* seedlings grown on defined nutrient media of varying K^+ concentrations. Representative 15-day-old Shandong *Eutrema* seedlings grown on 0 mM, 0.5 mM and 2 mM K^+. Seedlings showing apical region of primary roots with (A) no proliferation of long root hairs and (B) appearance of sporadic "tufts" of long root hairs on about 50% of seedlings. The magnification scale is given.


0 mM K+
0.5 mM K+
2 mM K+
A
B
1 mm
1 mm
1 mm
1 mm
1 mm
1 mm

DISCUSSION

Root systems of plants are highly plastic and their response to variations in the nutrient content of the environment is considered to be a critical factor in allowing plants to adjust their root growth to improve nutrient uptake (Kellermeier et al., 2013). As such, deficiencies in nutrients are often characterized by altered root architecture, a response that has been a topic of considerable interest to plant biologists (Lynch, 1995: Kellermeier et al., 2013: Gruber et al., 2013).

I. Conditions for K^+ deficiency testing

Studies of plants exposed to nutrient deficiencies have used soil-based media (Llorente et al., 2002: Kim et al., 1998) but this has not been the easiest medium to observe changes in root development. Rather, one of the more popular approaches applied to the study of root systems has been the use of gel-based media. Gels are colloids and, as such, are mostly liquid but perform like solids where both the composition of the gel and its firmness can be manipulated. Manipulating these properties yields a gel medium that supports growing roots, can be altered with respect to dissolved chemicals, and offers a relatively simple and effective way to visualize the spatial arrangement of roots. Changes in root morphology can also be monitored over time and in response to treatments such as exposure to specific nutrient deficiencies. However, this convenient experimental system is not without drawbacks. Primary among these drawbacks is that the agent used to cross-link the gel is seldom free of contaminating chemicals and both the extent and nature of the contaminating material(s)

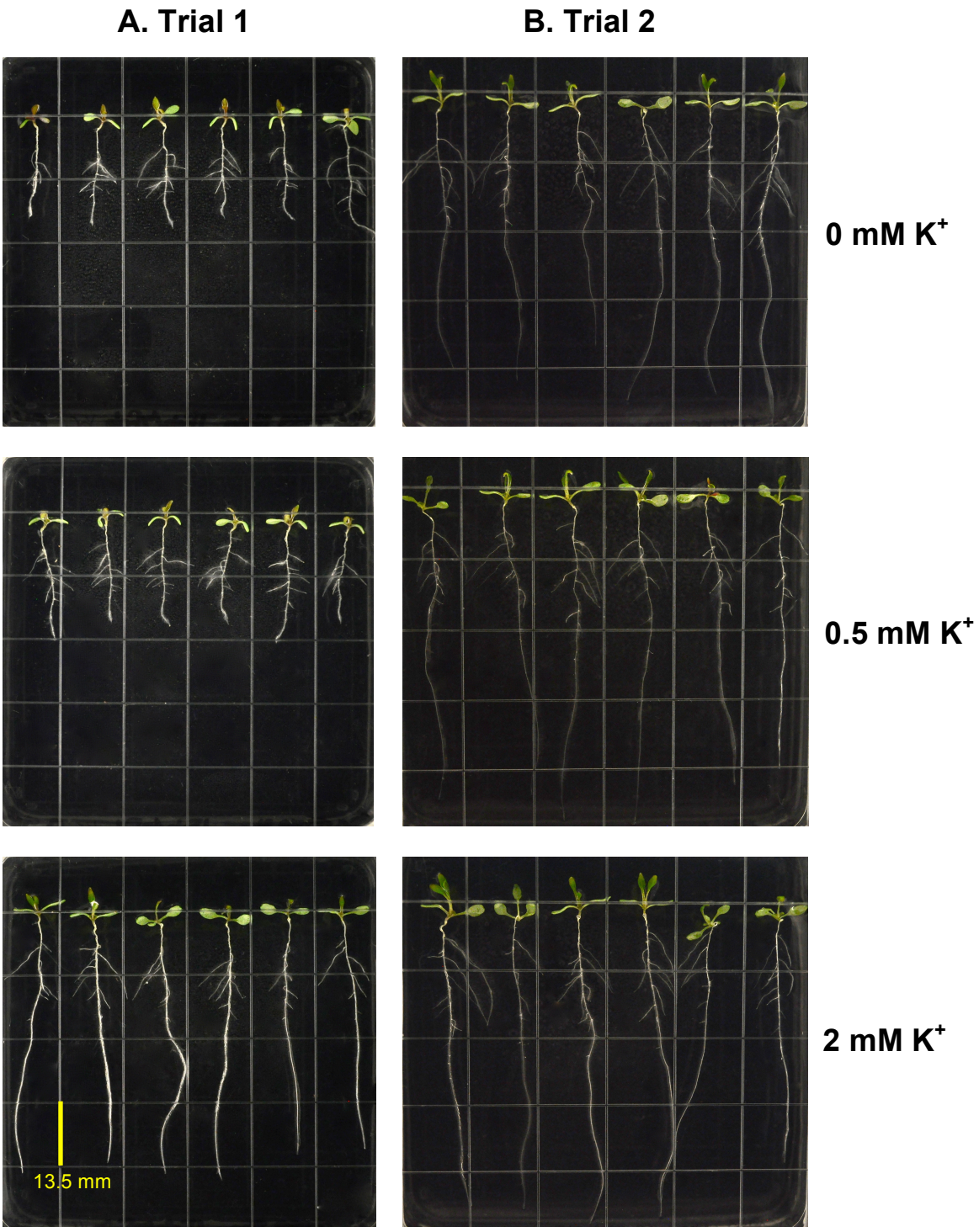
can contribute to inconsistent and flawed experimental results (Jain et al., 2009: Gruber et al., 2013).

Given the possible problems associated with gel-based media, it is surprising to learn from the literature that research laboratories studying the responses of plants to K^+ deficiency are seldom consistent with respect to selection and rigorous reporting on the type of gelling agent used. For example, Agullo et al. (2004) used Phytagel to solidify their culture medium whereas Armengaud et al. (2004) used agar and Qi et al. (2008) used agarose. This variation implies that virtually any choice of gelling agent is suitable for K^+ deficiency studies in plants.

There were two reasons why this work began with the selection of Phytagel as the gelling agent: 1) the report by Agullo et al. (2004) using Phytagel to study K^+ deficiency symptoms with *Arabidopsis*, and 2) our laboratory has used Phytagel successfully for several years to study *Eutrema* deficiencies related to nitrogen (Guevara et al., 2012) and phosphorous (V. Velasco, unpublished).

Initial results using Phytagel were promising in that one noteworthy and early experimental trial (Trial 1, Fig. 22) showed that Shandong *Eutrema* seedlings on Phytagel medium had a distinctly different root growth phenotype on 0.5 mM and 0 mM plates compared to 2.0 mM K^+ plates. Unfortunately, this result could not be replicated and all subsequent repeats of this experiment resembled Trial 2 in Figure 22 with all Shandong *Eutrema* plants growing equally well on the three K^+ levels tested. It is possible that Trial 1 was done on a different lot number of Phytagel than was used for later repeats of the same experiment. The significance of this detail was not recognized at the beginning of

Figure 22 - Two experimental trials comparing the growth response of Shandong *Eutrema* seedlings to varying K^+ using Phytagel as the gelling agent. Seven-day-old seedlings were grown in two separate experiments, (A) Trial 1 and (B) Trial 2. See Materials and Methods section for the details on germination and growth conditions including media formulation used for Trials. Lot numbers for the Phytagel product are not available for Trial 1 but Trial 2 used SLBB5682V.


A. Trial 1
B. Trial 2
0 mM K+
0.5 mM K+
2 mM K+
13.5 mm

this project.

To add additional uncertainty about the conditions needed to test for a K^+ deficiency, Armengaud et al. (2009) stated that "*Arabidopsis* plants grown in low K^+ were identical in growth and appearance to control plants during the first week of growth." This statement raised a question about the length of time needed to elicit a diagnostic K^+ deficiency phenotype so seedlings were then grown on large plates of Phytagel (245 x 245 mm) in order to allow the experiment to run for a longer period of time. Growing the seedlings on larger plates extended the length of the experiment by about 10 d, but even this change did not lead to any sign of a K^+ deficiency root response on nutrient plates solidified with Phytagel. Indeed, even *Arabidopsis* seedlings grown for 12 d on media lacking added K^+ did not differ in appearance to seedlings grown in the presence of added K^+ (data not shown). In a study of K^+ nutrition, Pandey et al. (2007) reported that *Arabidopsis* seedlings exposed to only four days of K^+ deficiency already show an almost 50% reduction in internal K^+ content relative to non-deficient controls. However, Figure 7 shows that *Arabidopsis* seedlings that I grew on 0 mM K^+ plates solidified with Phytagel had similar root K^+ content as seedlings grown in the presence of 0.5 or 2 mM K^+. Taken together, the lack of root phenotype and elevated root internal K^+ content led me to strongly suspect that there was a source of K^+ contamination present in the growth medium and that there was enough contaminating K^+ to prevent any deficiency of this macronutrient from developing in the seedlings that were being tested.

To address the question of K^+ contamination in the medium, an analysis of the K^+ content of all ingredients used to formulate the gel were performed using atomic

absorption spectrometry. This analysis indicated that the stock solutions comprising the mineral nutrient mix were relatively free of K^+ but the Phytagel used contained approximately 300-fold more K^+ than two other agar-based gelling agents that were also tested (Fig. 6). With this excessive level of K^+ present in the gelling agent, creating a deficiency for this nutrient to test plant responses was unachievable. Not surprisingly, there was no K^+ treatment-dependent difference in phenotype between the various seedlings grown on Phytagel (Fig. 3 and 4). Some insight into this problem was given by the work of Gruber et al. (2013) that was published as this project was underway. Gruber et al. (2013) reported on excessive K^+ levels being detected in Phytagel. Moreover, they found that interfering levels of K^+ were present in several different gelling agent sources with K^+ contamination levels varying 2000-fold. Their findings make it very clear that gelling agent choice is crucial for the accuracy and reproducibility of K^+ deficiency experiments and my results corroborate their contamination determinations for Phytagel and their conclusions. Fortunately, measurements of the K^+ content of the gels allowed for an informed selection of gelling agent and Sigma agar (A1296 - SLBF1721V) was used to generate a phenotypic comparison between the three plants used for this work.

II. Impact of K^+ deficiency on *Arabidopsis* and *Eutrema* root development

There have been conflicting reports in the literature on whether K^+ deficiency inhibits primary root growth in *Arabidopsis* (Shin and Schachtman, 2004; Jung et al., 2009; Kim et al., 2010). Shin and Schachtman (2004) showed that the primary roots of *Arabidopsis* seedlings were not impacted negatively by low K^+ whereas Jung and co-workers (2009) found that the extension of primary roots was inhibited on low relative to

high K^+ treatment conditions. Interestingly, both groups used the same levels of K^+ in their media and the same type of gelling agent (SeaKem agarose). The fact that both studies used agarose could explain the discrepancy in the reports since Gruber et al. (2013) reported that "*Arabidopsis* seedlings grew poorly or inconsistently on all of the agaroses tested." This further demonstrates how important the choice of gelling agent is on the reproducibility of K^+ deficiency experiments.

My results showed that there was a significant reduction in *Arabidopsis* primary root length when seedlings grew for 7 d on a medium lacking K^+ relative to seedlings on 2 mM K^+ (Fig. 11C) and that root extension was visibly delayed as early as Day 3 of the experiment (Fig. 10). Yukon *Eutrema* seedlings also had significantly reduced primary root growth when grown on media lacking added K^+ (Fig.10 and 13C) and the 1.8-fold decrease in primary root length observed at Day 10 was roughly the same magnitude as that found for *Arabidopsis* (Fig. 11C). Interestingly, the decrease in primary root length shown on 0 mM K^+ plates was greatest relative to Shandong *Eutrema* grown on 0.5 mM K^+ rather than 2 mM K^+ plates (Fig. 15C). The finding that Shandong seedlings grew best on 0.5 mM K^+ is consistent with K^+ at 2 mM having an inhibitory effect on root growth on Shandong but not Yukon *Eutrema* seedlings.

A classic K^+ deficient phenotype reported for *Arabidopsis* seedlings has been a reduction in lateral root proliferation (Shin & Schachtman, 2004: Jung et al., 2009: Kim et al., 2010). This result was also found for the combination of *Arabidopsis* and gel agar medium used in this study when no K^+ was added (Fig. 11D,E and F). Based on this outcome and the somewhat similar response with reduced growth of primary roots for 0

mM K^+ discussed above, I expected that *Eutrema* seedlings would show comparable root-related K^+ deficiency symptoms when grown on the gel-based media lacking K^+. Figures 13F and 15F show that both *Eutrema* accessions had significantly reduced lateral root growth on 0 mM K^+ agar plates although the approximate 1.6-fold reduction relative to seedlings on 2 mM K^+ plates was not as great as the 4-fold decrease found for *Arabidopsis*.

With respect to lateral roots, a noteworthy observation was that lateral root development on media lacking K^+ was, in general, less adversely impacted for *Eutrema* compared to *Arabidopsis* seedlings. For example, total lateral root length on K^+ deficient media for *Arabidopsis* seedlings was reduced by 86% relative to seedlings on 2 mM K^+ plates (Fig. 11F) whereas Yukon and Shandong *Eutrema* seedlings only showed a 40 to 46% reduction in total lateral root length (Fig. 13F and 15F). Furthermore, *Arabidopsis* seedlings showed a 75% decrease in the number of lateral roots on 0 mM relative to 2 mM K^+ plates (Fig. 11D) whereas Yukon and Shandong *Eutrema* seedlings only showed a 30 to 40% reduction for the same comparison (Fig. 13D and 15D).

We also found that root hair development was different depending on the plant and the K^+ content of the medium. For *Arabidopsis* seedlings, root hairs appeared to emerge from the primary root closer to the tip as the K^+ content of the media decreased (Fig. 19B). K^+-starved *Arabidopsis* seedlings are reported to undergo an increase in root hair elongation (Desbrosses et al., 2003: Shin & Schachtman, 2004) and our results support this finding based on visual observations (Fig. 19). Shandong *Eutrema* seedlings did not appear to show any K^+-specific changes in root hair morphology although

sporadic "tuffs" of longer root hairs occasionally appeared at the bottom of primary roots of seedlings on plates with 0, 0.5 and 2 mM K^+ (Fig. 21). The changes in root hair morphology for Yukon *Eutrema* seedlings that occurred on 0 mM K^+ plates were by comparison, remarkable. Root hairs of Yukon seedlings were consistently 2.75-fold longer when grown on 0 mM K^+ plates compared to seedlings grown in the presence of 0.5 mM or 2 mM K^+ (Fig. 20). Previous research with *Arabidopsis* has suggested that the root hair-specific accumulation of ROS during K^+ deficiency could serve as a signaling mechanism to the plant that soil K^+ levels have decreased (Desbrosses et al, 2003: Shin & Schachtman, 2004: Jung et al., 2009). Based on this theory, the root hair-specific increase in ROS levels could explain why Yukon *Eutrema* seedlings also have increased root hair length on K^+ deficient media, and this hypothesis could be an avenue of future research.

III. Impact of K^+ deficiency on *Arabidopsis* and *Eutrema* shoot biomass

For 10-day-old *Arabidopsis* and Yukon *Eutrema* seedlings, average shoot biomass for seedlings grown on 2 mM K^+ plates was approximately 4.0 mg ± 0.18 mg for both species. However when grown on media with no added K^+ (0 mM K^+), Yukon *Eutrema* seedlings had an average shoot biomass of 2.71 mg ± 0.09 mg, whereas *Arabidopsis* seedlings only averaged at 1.73 mg ± 0.10 mg. This represents a 62% decrease in shoot size for *Arabidopsis* plants between 2 mM K^+ and 0 mM K^+ plates, but only about a 30% decrease for Yukon *Eutrema* plants (Fig. 11A and 13A). Shandong *Eutrema* plants grown on media for 15 d had the highest shoot biomass on 0.5 mM K^+ plates at 6.51 mg ± 0.47 mg (Fig. 15A). When grown on 0 mM K^+ plates, Shandong *Eutrema* shoot biomass

averaged 3.42 mg ± 0.31 mg, representing a 47% decrease in shoot biomass that was comparable to the reduction observed for Yukon plants (Fig. 13A and 15A). These results indicate that *Arabidopsis* shoots were more severely affected by the lack of K^+ in the medium than *Eutrema* seedlings based upon the difference in fresh biomass measurements between seedlings grown on 0 mM K^+ and 2 mM K^+ plates (Fig.11A, 13A and 15A).

IV. K^+ content of seedlings

The K^+ content results indicate that *Arabidopsis* and *Eutrema* seedlings grown in the presence of K^+ (0.5 or 2 mM) contained significantly more K^+ in the roots and shoots than seedlings grown on 0 mM K^+ plates (Fig. 16, 17 and 18). Given that *Eutrema* seedlings experienced less severe growth inhibition on 0 mM K^+ media compared to seedlings grown on 2 mM K^+ plates than *Arabidopsis* (based on root measurements and biomass data), we anticipated that K^+-starved *Arabidopsis* tissue would have a lower extractable K^+ content relative to *Eutrema* tissue. Surprisingly, we found that both *Arabidopsis* and *Eutrema* seedlings grown on 0 mM K^+ plates showed a similar 79 to 85% lower root K^+ content than seedlings grown on 2 mM K^+ plates. That is, both *Eutrema* accessions had similar 5-fold lower root K^+ content when grown on 0 mM K^+ relative to 2 mM K^+ plates, a comparable difference to that found for *Arabidopsis* roots of seedlings grown on 0 mM K^+ relative to 2 mM K^+ (Fig. 16A, 17A and 18A).

The results we obtained for shoot K^+ content were even more surprising given our results for roots. Seedlings grown on 2 mM K^+ plates appeared to have maximal shoot K^+ levels at approximately 1.6 to 1.7 mmol K^+ g^{-1} DW for both *Arabidopsis* and *Eutrema*

(Fig. 16B, 17B and 18B). For 10-day-old *Arabidopsis* seedlings, the shoot K^+ content was 1.63 ± 0.30 mmol K^+ g^{-1} DW on plates with 2 mM K^+ but was only 0.66 ± 0.10 mmol g^{-1} DW when grown on plates with no added K^+ (0 mM K^+) (Fig. 16B). On the other hand, 10-day-old Yukon *Eutrema* shoot K^+ content was 1.72 ± 0.07 mmol K^+ g^{-1} DW on media with 2 mM K^+ and only 0.26 ± 0.03 mmol K^+ g^{-1} DW on 0 mM K^+ media (Fig. 17B). This represented a 6.6-fold decrease in shoot K^+ content for Yukon *Eutrema* seedlings on 2 mM K^+ verses 0 mM K^+ plates, but only a 2.5-fold decrease for *Arabidopsis* seedlings in the same comparison. Shandong *Eutrema* seedlings grown for 15 d showed an even greater difference in shoot K^+ content on 0 mM K^+ plates compared to 2 mM K^+ plates at 92%. Shandong *Eutrema* shoot K^+ content was 1.62 ± 0.12 mmol K^+ g^{-1} DW on 2 mM K^+ plates, and only 0.12 ± 0.01 mmol K^+ g^{-1} DW on media with no added K^+ (0 mM K^+), a change representing a 14-fold decrease between high and low K^+ (Fig. 18B). We found these results to be surprising in light of the similar differences we observed in the K^+ content of the roots discussed above. This raised a question about how the K^+ content we observed for *Arabidopsis* seedlings compared to reports in the literature. With differences in gel media and growth conditions used between laboratories a comparison with a matching set of conditions was impossible. However, by and large, the results we obtained for *Arabidopsis* shoot K^+ content are in agreement with those cited the literature. In terms of treatment effects, we found that *Arabidopsis* shoot K^+ content lower by 59% on 0 mM K plates compared to seedlings grown on 2 mM K^+ plates. In a study by Pandey et al. (2007), 11-day-old *Arabidopsis* shoots from seedlings on 0.02 mM KCl showed an approximate 50% decrease in shoot K^+ content

relative to seedlings on high K^+ medium, and Gruber et al. (2013) reported a 60% decrease in shoot K^+ content for 19-day-old Arabidopsis seedlings in a similar comparison. In both studies, shoot K^+ content was approximately 70 mg g^{-1} DW on high K^+ media and about 35 to 40 mg g^{-1} DW on low K^+ media. These results suggest that the ability of *Eutrema* seedlings to better cope with K^+ deficiency is unlikely to be due to a better capacity for K^+ uptake because they had less K^+ in their shoots but a higher shoot biomass. This observation is of interest in light of previous work investigating *Eutrema's* ability to cope with N-limiting conditions. Kant et al. (2008) found that Shandong *Eutrema* roots have a higher capacity for N-uptake under limiting conditions because 3-week-old N-starved *Eutrema* plants contained more internal N than similarly treated *Arabidopsis* plants. My results suggest that *Eutrema* does not take up more K^+, but rather the seedlings are just able to perform better despite a low availability of K^+ in the medium. How and the extent to which *Eutrema* seedlings tolerate the low K^+ medium would be an interesting topic for future research.

V. Comparison between responses of *Eutrema* accessions under high and low K^+

There were several differences shown between the two *Eutrema* accessions with respect to their comparative growth on 0 mM, 0.5 mM and 2 mM K^+ plates that are noteworthy. That is, while both *Eutrema* accessions grew better than *Arabidopsis* on the 0 mM K^+ plates based upon shoot and root biomass (Fig. 11A,B, 13A,B and 15A,B), there were important differences in how the *Eutrema* accessions responded to the variation in K^+ present in the plates. First, Yukon seedlings had significantly higher lateral root density on 0 mM compared to 2 mM K^+ plates whereas Shandong seedlings did not show

any significant change in lateral root density with K^+ (Fig. 13E and 15E). Second, Shandong seedlings showed similar root hair morphology on 0 mM K^+ plates as seedlings on 2 mM K^+ plates but Yukon seedlings consistently had much longer root hairs on 0 mM K^+ plates than seedlings on 0.5 or 2 mM K^+ plates (Fig. 20 and 21). Third, shoot and root biomass measurements showed that Shandong seedlings grew equally well on plates that contained 0.5 mM or 2 mM K^+ whereas Yukon plants consistently grew bigger in the presence of 2 mM K^+ compared to 0.5 mM K^+ (Fig. 12, 13A, B and Fig. 14 and Fig. 15A,B). In fact, Shandong seedlings grown on 0.5 mM K^+ plates had a significantly longer average combined primary root and lateral root length than seedlings grown on 2 mM K^+ plates (Fig. 15G), a response that was never observed for Yukon seedlings. The better overall growth of Shandong seedlings on 0.5 mM K^+ relative to 2 mM K^+ is interesting because it suggests that Shandong *Eutrema* seedlings are likely inhibited by the higher level of K^+ in the 2 mM K^+ plates.

SUMMARY

This research was conducted in order to document the responses of two accessions of *Eutrema* (Yukon and Shandong) to K^+ deficiency using the K^+ deficiency symptoms widely reported for *Arabidopsis* as a basis for comparison. The results presented here show that seedlings of both accessions of *Eutrema* were less negatively impacted by the lack of K^+ in the media than *Arabidopsis*. My research shows that root growth of Yukon and Shandong *Eutrema* seedlings was reduced on media lacking added K^+ and that this growth inhibition was less than the impact seen for *Arabidopsis* seedlings tested in parallel and grown under identical conditions. Other studies have identified *Eutrema* as better able to cope with severe nitrogen (0.4 mM N) deficiencies (Kant et al., 2008; Guevara et al., 2012) and phosphorus (0 mM) deficiencies (V. Velasco, unpublished data) compared to *Arabidopsis*. The quantitative measurements related to root growth under conditions deficient for K^+ presented here are consistent with *Eutrema* seedlings also being better equipped to cope with deficiencies in K^+ relative to *Arabidopsis*. Whether this improved seedling capacity to grow on low K^+ translates to producing mature plants with a better K^+-use- efficiency remains to be determined.

We also observed several differences between the two *Eutrema* accessions with respect to their comparative growth on 0 mM, 0.5 mM and 2 mM K^+ plates. The major differences observed related to lateral root length and density and root hair development and the extent to which these traits were altered by exposure of seedlings to a medium lacking K^+. The seemingly inhibitory response seen by Shandong seedlings exposed to 2 mM K^+ compared to 0.5 mM K^+ was also a feature that distinguished the two accessions

and the mechanism underlying this difference would be an interesting topic for future research.

In closing, plants, including halophytes, tolerate Na^+ better when K^+ is available but a favorable ratio of Na^+ to K^+ is important in improving plant performance as an imbalance of these ions can lead to injury (Ali et al., 2012). Given the fact that *Eutrema* is a halophyte capable of tolerating high levels of Na^+ while *Arabidopsis* is not, it is perhaps not surprising to see differences in the response of these species to variable K^+ in their environment. However, clearly there is something else at play to explain the differences between the *Eutrema* accessions with respect to the different K^+-related responses that they show. Those differences may reflect the soil conditions and the ratio of Na^+ and K^+ to which they have adapted or perhaps other biogeographical factors that remain to be discovered. With respect to soil, there are areas in the world where K^+ is present in such high quantities in soil that it is toxic for plant growth. An example of this is in the Pakistani mountains of Pashawa, where high levels of nitric acid and K^+ result in toxic levels of KNO_3 in the soil because the ratio of K^+ to Na^+ is much too high (Monk, 2004). The difference in the behavior of Shandong and Yukon seedlings revealed by this study using defined nutrient media plates may provide insight into the conditions under which these plants may have adapted to with respect to K^+ in their natural habitats rendering one accession seemingly more sensitive to elevated K^+ than the other.

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