

Photosynthetically active radiation is required for seedling growth promotion by volcanic dacitic tuff breccia (Azomite)

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Abstract

A plant's growth and development are shaped by its genome and the capacity to negotiate its environment for access to light, water, and nutrients. There is a vital need to understand the interactions between the plant, its physical environment, and the fertilizers used in agriculture. In this study, a commercially available volcanic ash fertilizer, Azomite[®], characterized as dacitic (rhyolitic) tuff breccia, was tested for its effect on promoting early seedling vigor. Early growth and photomorphogenesis processes are well studied in *Arabidopsis*. Seedling assays under different light conditions were used to dissect the underlying mechanisms involved. These assays are well established and can be translated to agriculturally important crop plants. The volcanic ash fertilizer was tested at different concentrations on seedlings grown on basic media lacking sucrose either in continuous darkness (Dc), continuous Red (Rc), Far-Red (FRc), or White Light (WLc). Micronutrients in the volcanic ash significantly increased seedling growth under Rc and WLc, but not under Dc and FRc, indicating that photosynthetically active radiation was required for the observed growth increase. Furthermore, red-light photoreceptor mutant, *phyB-9*, lacked the growth response, and higher amount of fertilizer reduced growth in all conditions tested. These data suggest that light triggers the ability of the seedling to utilize micronutrients in volcanic ash in a dose-dependent manner. The methods described here can be used to establish mechanisms of activity of various nutrient inputs and, coupled with whole-genome expression profiling, can lead to better insights into optimizing nutrient field applications to improve crop production.

KEYWORDS

micronutrients, photosynthesis, seedling growth, volcanic ash

1 | INTRODUCTION

To take advantage of the mineral nutrients present, volcanic ash-based fertilizers are applied in croplands, but dissecting the

mechanisms involved in promoting plant growth is challenging due to the complex compositions and unique characteristics of each deposit. Volcanic ash soils cover nearly 124 million hectares, about .84% of global land surface. Soils that are formed with volcanic ash, pumice,

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cinders, and lava develop colloidal fraction consisting of short-range-order minerals and active aluminosilicates (aluminum-humus). Weathering and mineral transformation under cool, humid climates generate andic soil properties, which is characteristic of Andisols, comprising 60% or more of their upper layer thickness with allophane, imogolite, and ferrihydrite (Soil Survey Staff, 1999). Andisols have low bulk density, high phosphate retention, and high water-holding capacity (Shoji & Ono, 1978; Takahashi & Shoji, 2002), which leads to the accumulation of organic carbon and nitrogen, making these soils one of the most productive soils in the world with long-term agricultural and environmental sustainability (Shoji & Takahashi, 2002), as evidenced by the rapid revegetation of volcanic ash deposits in areas of Mt. Usu in Japan, Mt. Pinatubo in the Philippines, and Mt. St. Helens in the United States.

Two patterns of revegetation have been reported depending on the thickness (amount) of the deposit: (a) establishment of seedlings on thick (1–3 m) ash deposit by wind-dispersed seeds of trees or emergence of gramineous and leguminous plants and (b) by resprouting of buried vegetation on thin (<1 m) ash deposit (Haruki & Tsuyuzaki, 2001; Saito et al., 2002; Shoji & Takahashi, 2002). These studies suggest that fertigation of agricultural crops with volcanic ash can provide benefits even early during seed germination. Each volcanic ash deposit has widely different structure and composition. Therefore, it is important to understand the underlying mechanisms involved in plant responses to different volcanic ash fertilizers, including at the stages of early seed establishment.

In flowering plants, the seed protects and supports the mature embryo for its survival. Seeds remain dormant until the conditions become favorable for germination and provide nutrients to support early seedling growth (Koornneef & Karssen, 1994). Seedlings growing in the subterranean darkness of soil undergo skotomorphogenesis or etiolation, a process distinct from seedling development in light or photomorphogenesis (Von Arnim & Deng, 1996). Etiolation is achieved by cell elongation in the hypocotyl, while the folded shoot meristem is protected by the apical hook curvature as the seedling grows through the soil to reach sufficient light for photoautotrophic growth (Von Arnim & Deng, 1996). This mechanism is conserved across vascular plants to prevent photooxidative damage, which is phototoxic to the seedling caused by premature light exposure of protochlorophyllide, a precursor of chlorophyll (Reinbothe et al., 1996).

Exposure to light triggers photomorphogenesis, which is marked by the inhibition of hypocotyl elongation, opening of the apical hook, cotyledon unfolding, and initiation of chlorophyll biosynthesis (greening) (Quail, 2002a). During this process, the seedling exhibits precisely orchestrated photosensitivity to optimize its growth and development under the incumbent conditions. This is achieved through the perception of light as a signal, which carries information about color, direction, intensity, and duration of light. In *Arabidopsis*, the magnitude of light inhibition of hypocotyl length is synchronized with the reciprocal increase in cotyledon area during photomorphogenesis (Quail, 2002a). Previously, this correlation has been used as a diagnostic criterion to reveal that only a small number of early light-regulated genes are necessary for optimal de-etiolation (Khanna et al., 2006). The relationship

between hypocotyl length and cotyledon area in the wild type seedlings was set to unity under set light conditions, and 32 genes categorized as early light responsive from microarray profiles were selected for analysis using corresponding gene-mutant lines (Khanna et al., 2006). Only 7 out of 32 gene lesions resulted in aberrant de-etiolation phenotypes, characterized by strict reciprocal correlations exhibiting increased hypocotyl length and reduced cotyledon area (hyposensitive to light), or inhibited hypocotyl length with enlarged cotyledon area (hypersensitive to light), relative to the wild type under the given light conditions (Khanna et al., 2006). These studies identified new genetic lesions that disrupted normal progression of photomorphogenesis. About half of the remaining mutant lines displayed no significant phenotypes. These genes were determined to be responsive to light but played either redundant or indirect roles in early seedling development. Whereas several other mutant lines exhibited three different responses, either parallel inhibition or parallel enhancement of cell expansion in both hypocotyl and cotyledon, or only hypocotyl inhibition with no significant effect on cotyledon area (Khanna et al., 2006). This latter set of genes are more likely to be affected in general cell growth processes either in one or both organs but are not directly involved in the process of photomorphogenesis.

During photomorphogenesis, early seedling establishment is subjected to other environmental factors, including temperature and the availability of water and nutrients. A complex intersection of light responses with responsiveness to other environmental conditions collectively shapes the seedling's growth in the soil. Sensitivity to informational light signal is achieved through a collection of photoreceptors with some overlapping and some distinct functions. *Arabidopsis* seedlings monitor light wavelengths through multiple photoreceptor systems, which include five phytochromes (phyA-phyE) for red/far-red (R/FR) and some degree of blue (B) light, along with cryptochromes (cry1 and cry2), the Zeitlupe (ZTL) family and phototropins (phot1 and phot2) for blue/UV-A, and UV Resistance Locus 8 (UVR8) for UV-B light (Briggs & Christie, 2002; Briggs & Huala, 1999; Chen et al., 2004; Quail, 2002a, 2002b; Rizzini et al., 2011). Plants can sense light radiation ranging from UV-B to FR (280–800 nm), which includes photosynthetically active radiation (PAR, 400–700 nm) (McCree, 1973). Photosynthetic efficiency is highest under red photons (600–700 nm), followed by green (500–600 nm) and blue (400–500 nm) photons (Inada, 1976), and it drops rapidly under wavelengths longer than 685 nm extending into the FR range, known as the “red drop” (Emerson & Lewis, 1943). However, FR and PAR photons can act synergistically under simultaneous irradiation (Emerson et al., 1957; Zhen et al., 2021, 2022). As the seedling becomes photoautotrophic, its rate of growth is shaped by the degree of photosynthesis and transpiration under daily variations in temperature, humidity, and soil composition.

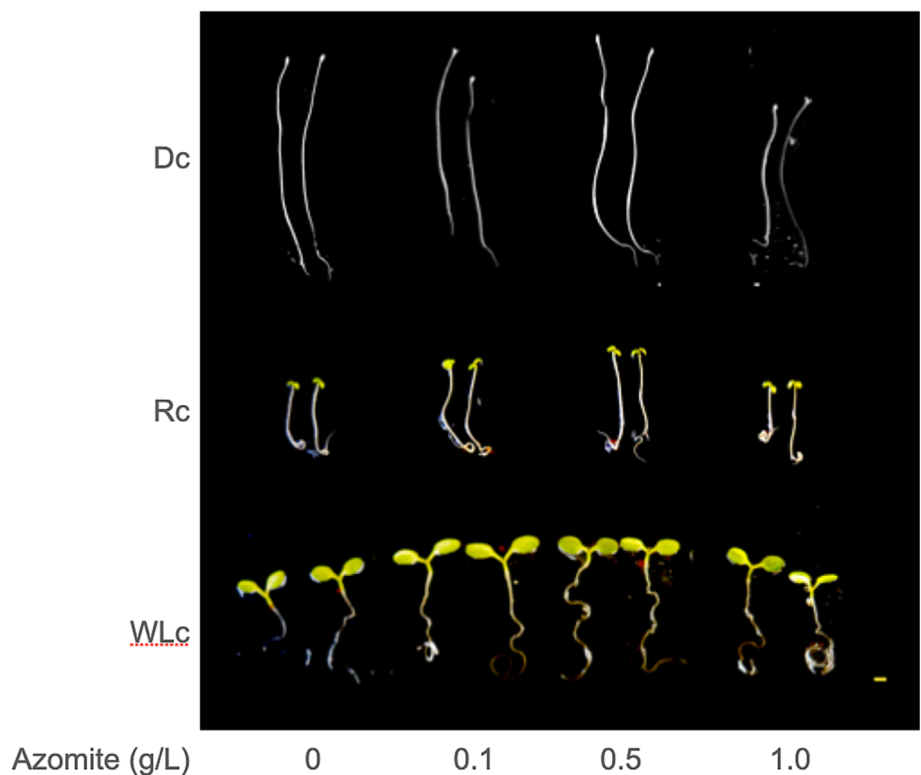
In addition to light, early seedling establishment is highly sensitive to concentrations of cellular metabolites such as sugars and amino acids. Sugars provide intermediary source of metabolism and act as signaling molecules (Rolland et al., 2006). In the dark, the embryo transitions from mitotic growth to differentiation and cell expansion, marked by increased sucrose uptake derived from the stored neutral

lipids and triacylglycerols (Pyc et al., 2017), accompanied by changes in gene expression and the activities of metabolic enzymes (Weber et al., 2005). Seedling growth in darkness through the soil is further supported by a sucrose feedback loop to increase sucrose levels in cotyledons (Mu et al., 2022). Cotyledon greening is triggered by light through the suppression of constitutively photomorphogenic 1, and it is facilitated by phytochrome interacting factors through the suppression of protochlorophyllide accumulation in darkness (Duek & Fankhauser, 2005; Zhong et al., 2009). FR light inhibits greening by suppressing the light-dependent conversion of protochlorophyllide A to chlorophyllide A, which is required for chlorophyll biosynthesis (Barnes et al., 1996). Exogenous application of sucrose can remove this FR block of cotyledon greening (Barnes et al., 1996), suggesting that sugars promote greening. Concomitant assembly of chlorophylls along with proteins and lipids leads to thylakoid and chloroplast differentiation (Jarvis & Lopez-Juez, 2013). Chloroplast development can be divided into two distinct phases, namely, structure establishment and chloroplast proliferation (Pipitone et al., 2021). These stages of development rely upon several integral components, such as the insertion of Mg^{2+} into protoporphyrin IX by Mg^{2+} -chelatase, a two-step process that is tightly regulated by light, the circadian clock, and the photosynthetic electron transport, involving regulation at transcriptional and post-transcriptional levels (Masuda, 2008).

The switch from cell expansion growth in darkness to early seedling establishment and subsequent growth is achieved through a continuum of signaling and resource management. The underlying molecular components of photomorphogenesis are well studied and described to be regulated at the levels of transcriptome and proteome

in response to environmental cues. Knowledge of these mechanisms can be applied to dissect molecular pathways related to plant fertilizers. We tested a highly mineralized complex silica ore volcanic ash, surface mined in Utah, Azomite (AZOMITE® Mineral Products, Inc.) because of its sustainability potential as a fertilizer sourced from a naturally occurring volcanic deposit. In a previous study, it was found that Azomite volcanic ash (dacitic [rhyolitic] tuff breccia; DTB) increased tomato fruit production, and concurrently, it modified the rhizosphere and root endosphere microbial community architecture (Mehlferber et al., 2022). We hypothesized that the temporal changes in microbiome community structures were primarily driven by the nutrient substrates available to the microbial communities and that the observed shift suggested an increase in higher carbon (from C2 to C6) compounds in the host plant endosphere and possibly root exudates (Mehlferber et al., 2022). These results suggested that Azomite treatment somehow modified host plant nutrient profile thereby causing a microbiome shift. Further, these changes pointed toward a plausible increase in the accumulation of photosynthesis products. Based on these findings, we wanted to test whether micronutrients in form of DBT impact plant growth directly in a nutrient-free medium. To investigate this possibility, we utilized a well-established *Arabidopsis* seedling photomorphogenesis test for two reasons: one to unravel the mechanism of action and second to develop a reliable bioassay for any similar fertilizer. Here, we show that Azomite promotes seedling growth in *Arabidopsis* during photomorphogenesis in a dose-dependent manner, and its activity is PAR-dependent. Deeper understanding of these mechanisms is essential for the development of safe and environmentally sustainable fertilizers.

FIGURE 1 Images of *Arabidopsis* seedlings treated with increasing Azomite concentrations under light or darkness. Representative images of Col-0 seedlings grown for 4 days in continuous darkness (Dc), red light (Rc), or white light (WLC) on media plates without sucrose, supplemented with different concentration of Azomite ultrafine (volcanic ash) as indicated.



2 | RESULTS

2.1 | Azomite micronutrients promote early seedling growth

In this study, we used the previously developed seedling deetiolation assay to dissect the mode of action of a commercially available fertilizer, Azomite. Arabidopsis seedlings grown under Dc, Rc, or WLC for 4 days responded differentially to increasing amounts of Azomite in the growth medium, as seen in Figure 1. Both, hypocotyl length and cotyledon area measurements revealed a similar direction of change in growth under Rc and WLC (Figures 2 and 3), indicating that Azomite caused changes in general seedling growth, and it did not impact photoreceptor function, which would have been marked by a reciprocal hypocotyl and cotyledon growth response. There was a pattern of increased hypocotyl length (20%) in response to .5 g/L of Azomite in Rc and .1 g/L in WLC (Figure 2). However, higher concentrations of Azomite, under all conditions tested, including Dc, Rc, and WLC led to relatively reduced hypocotyl growth (Figure 2). We also noted a significant increase in cotyledon area, 20% in Rc with .5 g/L, and 25% in WLC with .1 g/L Azomite, and this effect on cotyledon area was reduced under higher concentration tested (Figure 3). These initial results indicated that Azomite increased both hypocotyl elongation and cotyledon area under Rc

and WLC in a dose-dependent manner (Figures 2 and 3), but there was no significant effect on etiolated seedlings (Figure 2). In addition, the peak increase in seedling growth under WLC was attained at lower amounts of Azomite compared with the peak increase in growth under Rc (Figures 2 and 3).

Next, we tested photoreceptor *phyA-211* and *phyB-9* mutants under Rc and FRc light with Azomite treatments to test whether altered photosensitivity to red or far-red light was linked to the observed response. As expected, *phyA-211* seedlings were insensitive to FRc, and *phyB-9* seedlings showed reduced Rc sensitivity and Col-0 responded normally to the various light treatments (Figure 4). There was no significant effect of Azomite treatments under Dc or FRc conditions on any of the genotypes tested (Figure 5). There was a significant change in hypocotyl length elongation in Col-0 mutants in response to .5 g/L Azomite under Rc, exhibiting up to 20% increase in growth, a trend that was repeated, albeit not reaching significance in the *phyA-211* mutants (containing functional *phyB*), which exhibited an increase by 10% (Figure 5). While all five Arabidopsis phytochromes absorb in the red region, *phyB* is the dominant contributor in hypocotyl length response under red light (Tepperman et al., 2004), and *phyA* regulates growth under far-red light (Wang et al., 2002). These data indicated that photosensitivity to photosynthetically active red light was required and that far-red or dark conditions were insufficient for Azomite activity on seedling growth.

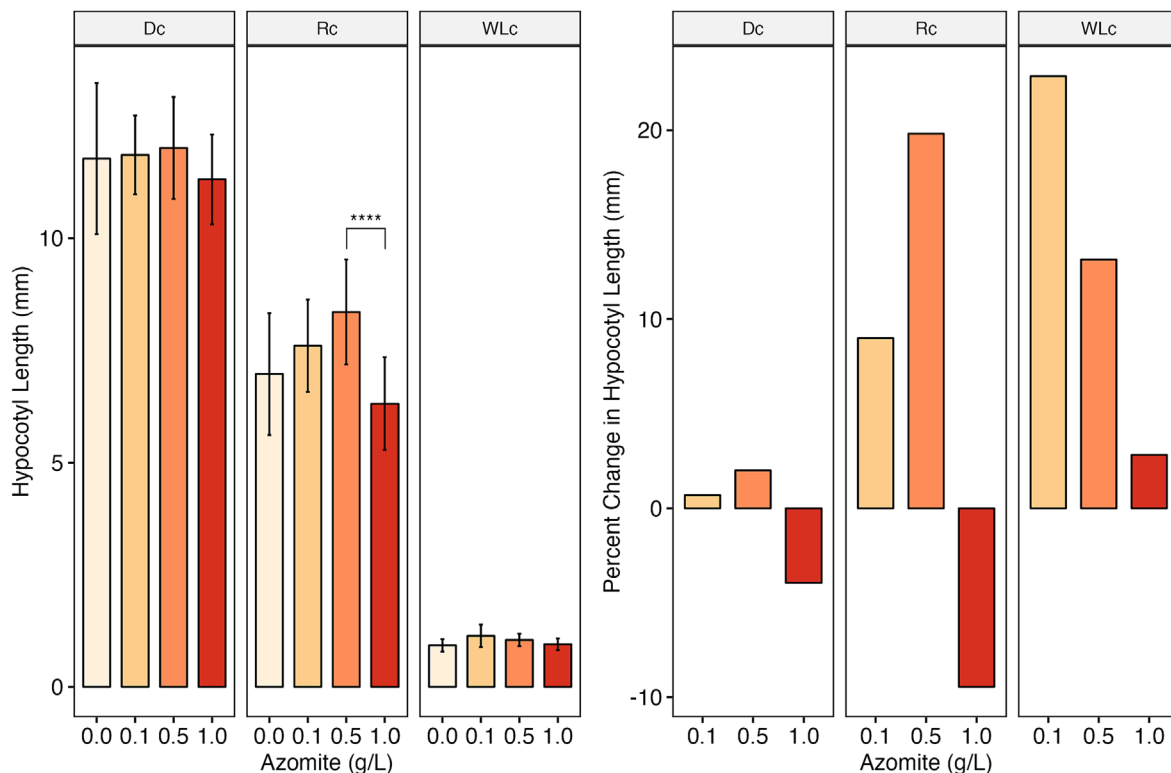


FIGURE 2 Quantitative comparison of hypocotyl length under red and white light. Mean values of hypocotyl length of 4-day-old Col-0 seedlings in response to treatments with indicated Azomite concentrations grown in Dc, Rc, or WLC (left). Percent change in hypocotyl length is shown for Azomite-treated seedlings, relative to untreated (0 g/L) control (right). SE are plotted; *p*-values adjusted for multiple testing comparisons are represented as asterisks. *****p*-value < .0001.

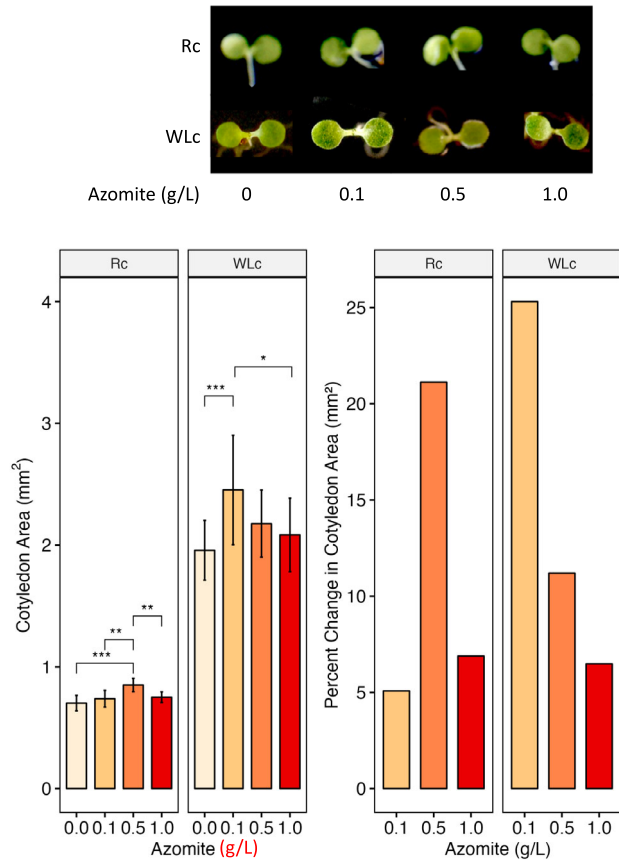


FIGURE 3 Cotyledon and hypocotyl responses to Azomite under red and white light. Representative images of cotyledons of 4-d-old Col-0 seedlings are shown in response to treatment with indicated Azomite concentrations under Rc and WLC (top). Mean values of cotyledon area of seedlings treated with different Azomite concentrations are plotted (left). Percent change in cotyledon area in response to Azomite treatment, relative to untreated (0 g/L) control (right). SE are plotted; p -values adjusted for multiple testing comparisons are represented as asterisks, with * $p < .05$, ** $p < .01$, and *** $p < .001$.

Relative mean values of hypocotyl length and cotyledon area were plotted for Col-0 grown under Rc or WLC with different amounts of Azomite as indicated (Figure 6). Average growth measurements of Col-0 were set to 1 for hypocotyl length and cotyledon area as previously described (Khanna et al., 2006). In both Rc and WLC, increasing levels of Azomite concentration move the hypocotyl length/cotyledon area measurements closer toward increased general growth, with a concentration of .1 g of Azomite maximally increasing plant growth under WLC and .5 g of Azomite maximally increasing growth under Rc. However, past these optima, general growth decreases, and in Rc at 1.0 g of Azomite, the hypocotyl length is shortened more relative to cotyledon area, moving the seedlings toward a hypersensitive response. Here, we can draw two conclusions: one that de-etiolating seedlings exhibit dose-dependent sensitivity to volcanic ash composition and second that Azomite activity in seedlings interacts with light-regulated development.

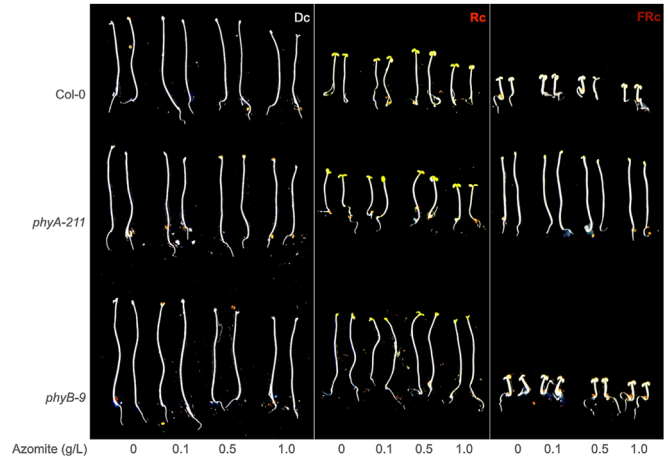


FIGURE 4 Images of Arabidopsis seedlings treated with increasing Azomite concentrations under darkness, red, or far-red light. Representative Col-0 and phytochrome mutant (*phyA-211* and *phyB-9*) seedlings grown for 4-days in Dc, Rc, or FRC, and treated with indicated Azomite concentrations are shown.

2.2 | PAR is required for Azomite induced early seedling growth

While relatively lower concentrations of Azomite increased hypocotyl and cotyledon growth under Rc and WLC, there was no significant effect of Azomite treatment on seedling growth under Dc and FRC (Figures 2 and 5), indicating that Azomite activity required photosynthetically active radiation. These results were unexpected and suggest that during early seedling establishment, activation of the photosynthetic circuitry precedes seedling utilization of certain micronutrients, such as those present in volcanic ash product Azomite. Data represented in Figure 6 show that under optimal concentrations, Azomite can promote growth processes in both organs, which is likely to involve modifications in gene expression under the conditions tested.

3 | DISCUSSION

The addition of Azomite (a volcanic ash dacitic tuff breccia) used with MS salts lacking sucrose, increased growth of photomorphogenic seedlings, but not of etiolated or FRC grown seedlings. Early seedling growth is dependent upon environmental factors and nutrient availability. The unexpected observation that under controlled environments, Azomite effect on seedling growth required photosynthetically active radiation, suggests that one or more of Azomite components may be involved in promoting photosynthesis-related growth regulating processes during deetiolation. Normal deetiolation is characterized by concomitant inhibition of hypocotyl and stimulation of cotyledon growth (Khanna et al., 2006). In contrast, Azomite application stimulated both hypocotyl and cotyledon growth in a dose- and light-wavelength-dependent manner, and higher amounts of Azomite had a growth inhibitory effect. These data provide several novel insights

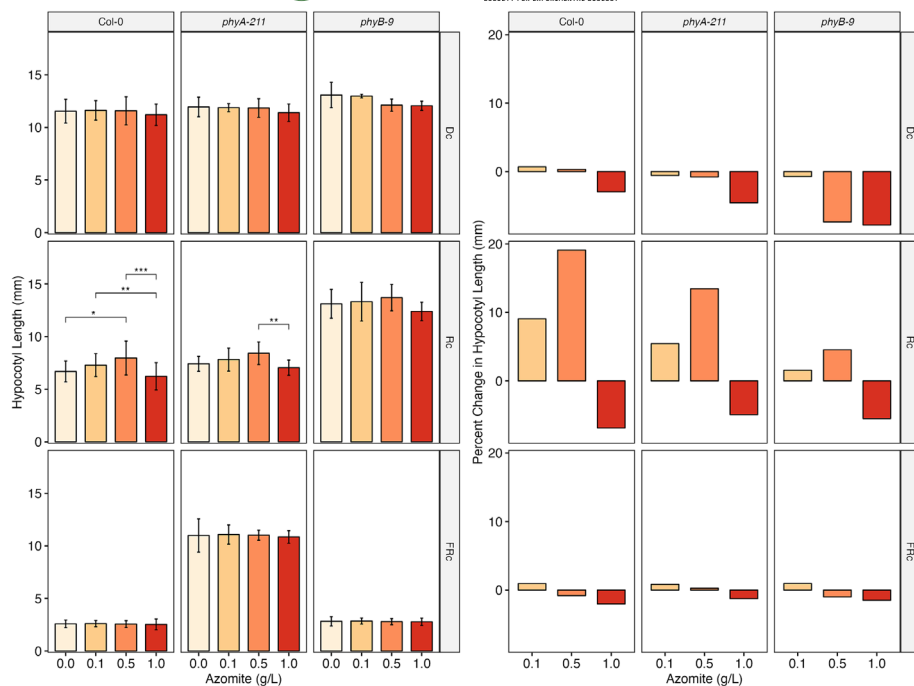


FIGURE 5 Quantitative comparison of hypocotyl length under darkness, red, or far-red light. Mean value of hypocotyl length of 4-day-old Col-0 and phytochrome mutant (*phyA-211* and *phyB-9*) seedlings grown for 4-days in Dc, Rc, or FRc are plotted with corresponding Azomite treatments (left). Percent change in hypocotyl length relative to untreated (0 g/L) control is shown for seedlings treated with different Azomite concentrations and light conditions (right). SE are plotted; *p*-values adjusted for multiple testing comparisons are represented as asterisks. **p* < .05, ***p* < .01, and ****p* < .001.

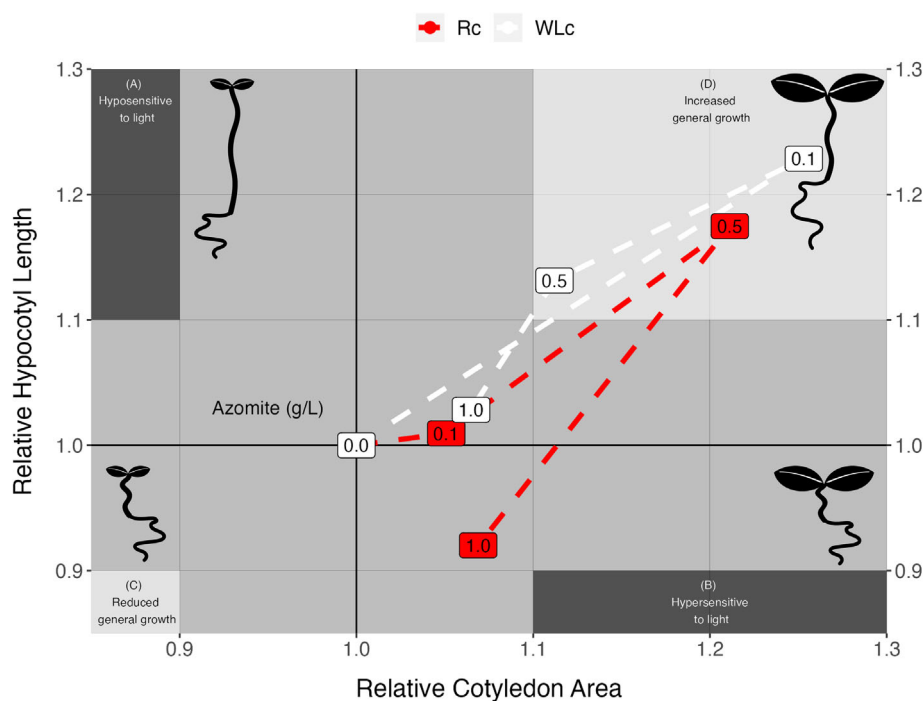


FIGURE 6 Comparison of relative changes in cotyledon area and hypocotyl length in response to Azomite under red and white light. Azomite induced seedling phenotypes. Relative mean values of hypocotyl length were plotted against relative mean values of cotyledon area, with control (0 g/L) values set to unity. The quadrants represent previously described relationships of cell elongation phenotypes displayed by the two organs during deetiolation. (A) Hypersensitive, reduced light sensitivity. (B) Hypersensitive, increased light sensitivity. (C) Reduced general growth. (D) Increased general growth (Khanna et al., 2006). Normal deetiolation is represented at the point of cross-section between the quadrants (.0, control).

into the possible mechanisms underlying the growth effects of Azomite on plant growth and development.

3.1 | Possible mechanisms of Azomite activity

At present, it is unknown how volcanic ash's wide complex of mineral or element component/s enhance plant growth. The requirement of PAR suggests a shift in the seedling's ability to mobilize the active

ingredients. Previous studies have shown that light alters plant nutrient dynamics. Ockenden and Lott (1987) showed that when grown in distilled water, etiolated *Cucurbita* seedlings exported more minerals from cotyledons to the root-shoot axis than light-grown seedlings, whereas when grown under light, in soil with sufficient nutrients, the fully expanded, photosynthetic cotyledons had increased K by 5-fold and Ca by 50-fold compared with the cotyledons of dry embryos. Even in distilled water conditions, cotyledons of light grown seedlings accumulated 17% more Mg and 36% more P compared with those of



dark-grown seedlings (Ockenden & Lott, 1987). Cucurbit cotyledons green early during deetiolation, and exhibited low export of Mg from cucurbit cotyledons, which could be related to the requirement of Mg in chlorophyll biosynthesis in the cotyledons in response to light (Ockenden & Lott, 1987). Light has been shown to influence symbiotic nitrogen fixation more than soil-extractable nitrogen available to the plant (Taylor & Menge, 2018), demonstrating the complexity of how light impacts nutrient assimilation. Light quality, intensity, and photoperiod have been shown to have some overlapping and distinct influences in regulating uptake and utilization of different crop nutrients (Xu et al., 2021). These studies are aligned with our findings that light plays a critical role in fertilizer efficacy.

The deetioliating seedling assay revealed that PAR (not far-red light) was required for Azomite activity, thereby suggesting a role for photosynthesis. The DTB ash contains Mg, which could influence chlorophyll production in light grown seedlings. Studies are under way to examine this possibility. The amount of plant-available P in andisols (Truog-extractable P_2O_5) is similar to the amount found in fresh volcanic ash (Shoji & Takahashi, 2002), implying that majority of extractable P is plant-available. P deficiency has been shown to effect absorption of mineral nutrients and photosynthetic performance in citrus seedlings (Meng et al., 2021). A possible role of P in the Azomite ash product cannot be ruled out. During deetiolation, seedlings transition to autotrophic growth, and it is possible that inclusion of Azomite minerals in the growth medium accelerated mineral mobilization under PAR.

In addition to differential nutrient remobilization between organs of etiolated and green seedlings, the mineral elements may have a regulatory function. For example, K transport into guard cells is directly related to light-regulated stomatal opening (Humble & Raschke, 1971). Both deficiency and excess of K reduced nitrogen absorption and carbon assimilation and inhibited growth and root development in apple dwarf rootstock seedlings, suggesting that optimal K levels are required for transport of photosynthetic products from leaves to roots and nitrate transport and assimilation (Xu et al., 2020). Import of Ca into the chloroplast is light-dependent and has been linked to regulation of photosynthesis, possibly through changes in the activities of Ca^{2+} -binding proteins involved in photosynthesis electron transfer and photo-protection (Hochmal et al., 2015; Weinl et al., 2008). In maize seedlings during deetiolation, only 7% of proteins significantly changed in abundance, while there was a 26.6% change in phosphorylation status, including phosphoproteins involved in gene regulation and rate-limiting steps in light and carbon reactions of photosynthesis (Gao et al., 2020). It is conceivable that the observed PAR-dependent seedling growth promotion by Azomite was mediated through downstream regulation of photosynthesis-related processes, including gene expression, post translational modification, and regulation of enzymes and transport channels. Further studies are being conducted to test these possibilities.

At the higher concentration (1 g/L) tested, Azomite reduced cotyledon expansion and hypocotyl growth in etiolated, FRc and PAR-grown seedlings (Figures 6), implying an optimal range ($>.1$ to <1.0 g/L) of

beneficial Azomite activity on Arabidopsis seedlings during deetiolation. Recent experiments performed with greenhouse grown tomatoes have revealed similar relationships between plant growth and Azomite concentrations (Mehlferber et al., 2023). Higher concentrations may result in an increase in growth inhibition, potentially due to currently unknown impacts of excessive nutrient elements like K, as described above (Xu et al., 2020). These data suggest that the application of volcanic ash fertilizers, like Azomite may increase soil productivity similarly to that seen in areas of natural volcanic soil accumulation (Shoji & Takahashi, 2002). It is anticipated that the optimal concentrations will depend upon several factors, including plant size, rate of photosynthesis, other soil-borne factors, and environmental conditions. Overall, at the effective concentrations, volcanic ash-based fertilizers can offer an environmentally sustainable option to promote plant growth and reduce the reliance on traditional fertilization practices.

The measured peak of Azomite's beneficial activity shifted to lower Azomite concentrations under WLC (at .1 g/L), compared with Rc (.5 g/L) (Figure 6). This could be due to the monochromatic red (660 nm) light used compared with white light (400–700 nm) with a broader range of PAR. However, this observation suggests an intricate relationship between the seedling's photosynthetic capacity and available concentration of Azomite volcanic ash in the medium.

Light exerts its influence on plant growth and development directly and indirectly through the regulation of the circadian clock (Oakenfull & Davis, 2017). In a previous study (Khanna et al., 2006) with 32 independent gene mutant lines, each corresponding to genes rapidly and robustly induced by phy-mediated light signal, and encoding putative transcription factors, signaling components, and proteins of unknown function, only one mutant line with a T-DNA insertion in a MYB family transcription factor was found to display significant parallel enhancement of both hypocotyl and cotyledon expansion, similar to the phenotypes presented here with optimal Azomite concentrations (Figure 6). Subsequently, this locus was identified as *CIRCADIAN1/REVEILLE2* (*CIR1/RVE2*), belonging to a family of 11 proteins, and implicated in disrupting circadian clock-regulated gene expression (Chaudhury et al., 1999; Zhang et al., 2007). The parallel growth enhancement of both hypocotyl and cotyledons is likely to be mediated through altered expression of genes in response to Azomite. It remains to be seen whether gene expression of components intrinsic to the circadian clock is altered by Azomite, which may influence clock outputs, such as plant growth, development, and photosynthetic capacity. These studies will have broader implications in understanding the beneficial effects of volcanic ash and molecular processes involved in revegetation of regions impacted by volcanic deposits.

4 | CONCLUSIONS

In this study, we determined that the addition of Azomite, a DTB, promoted early seedling growth, in Rc and WLC. Absence of phyB function abrogated the growth response, whereas lack of phyA activity (in presence of active phyB) reduced growth promotion. phyB is known

to play a major role in regulating hypocotyl growth under Rc. Azomite-stimulated growth promotion was absent under FRc and in darkness, indicating a dependence on PAR, which in turn suggests a role of photosynthesis in Azomite promoted plant growth. Several possible underlying mechanisms are proposed for Azomite mode of action. It is anticipated that Azomite influences growth through changes in plant gene expression. These studies are currently underway.

The commercial fertilizer market was estimated to be over USD 190 billion in 2020, and it is expected to grow at a CAGR of 2.6% by 2030 (Global Market Insights, Inc., DE). There are many different types of fertilizers available in the market. Most of these products are tested on the field, with little to no insight into their mechanism of activity. The method presented here provides a convenient seedling assay to quantitatively test different fertilizers for their potential effects on plant growth and development. Deetiolation in *Arabidopsis* seedlings is highly sensitive to alterations in growth conditions, and it is one of the most studied phenomena at genetic, molecular and biochemical levels, providing access to established knowledge for data interpretation and tools, such as mutant lines for further investigation. Understanding how and why specific fertilizers and plant amendments work is critical to improving their efficacy and predicting how they may interact when used in tandem, as we work to improve crop yield and sustainability.

5 | MATERIALS AND METHODS

5.1 | Plant materials and growth conditions

Arabidopsis thaliana Columbia ecotype (Col-0), *phyA-211*, or *phyB-9* seeds were sterilized (Khanna et al., 2006) and plated on half-strength MS (Murashige & Skoog Basal Salt Mixture, PhytoTechnology Laboratories) salts, buffered with 1 g/L MES (2-(N-Morpholino) Ethane Sulfonic Acid, Research Organics, Inc.) at pH 5.8 with KOH, plus .7% Agar Noble (Difco laboratories). The media did not contain any added sugars. Azomite ultrafine (Azomite Mineral Products, Inc.) was added at various concentrations: 0, .1, .5, and 1.0 mg/mL prior to autoclaving, and Azomite was maintained as a uniform suspension in the media by shaking while pouring the plates. The experiments were repeated three to five times consistently by stratifying the plates for 4 days at 4°C; germination was induced by 3 h of white light treatment in a growth chamber at 21°C, followed by continuous placement in the growth chamber in darkness for 21 h. The plates were then transferred to respective light treatments in four different growth chambers at 21°C for 4 days for seedling growth either in Dc, WLC (5 $\mu\text{mol m}^{-2} \text{s}^{-1}$), Rc (660 nm, 7 $\mu\text{mol m}^{-2} \text{s}^{-1}$), and FRc (740 nm, 2 $\mu\text{mol m}^{-2} \text{s}^{-1}$), as indicated. Fluence rates were monitored using a spectroradiometer (model L1-1800; LICOR). Figure 1 shows 4-day-old seedlings grown in continuous darkness (Dc), continuous red light (Rc), or continuous white light (WLC). The initial light treatments were selected to assess any observable phenotypic differences in response to Azomite concentrations, which were chosen based on relative amounts used in previous greenhouse tomato trials (Mehlferber et al., 2023).

5.2 | Experimental design and treatments

In total, three sets of experiments were performed. In the first, hypocotyl length was measured in Col-0 *Arabidopsis* seedlings treated with either 0, .1, .5, and 1.0 mg/ml of Azomite, and subsequently grown under either Dc, WLC, or Rc conditions. In this experiment, 20 plants were started per treatment, but a variable number of seedlings germinated, leading to an imbalanced number of replicates per treatment, varying between 9 and 20. In the second experiment, cotyledon area was measured in Col-0 Azomite seedlings treated with 0, .1, .5, and 1.0 mg/ml of Azomite, and then grown under WLC or Rc conditions. In total, 30 plants were started for the WLC condition, while 15 were started for the Rc conditions, again, a variable number of seedlings germinated, ranging from 22 to 26 seedlings per treatment under the WLC condition, and 8 to 12 seedlings per treatment in the Rc condition. In the final experiment, hypocotyl length was again measured, this time across Col-0, *phyB-9* and *phyA-211* plants treated with either 0, .1, .5, and 1.0 of Azomite, and subsequently grown under either Dc, Rc, or FRc conditions. In this experiment, 35 plants were started per treatment, with a variable number germinating across each line. These replicates varied across Azomite and light treatments with 17 to 34 plants in the Col-0 line, with 6 to 35 for the *phyB-9* line, and 5 to 24 in the *phyA-211* line. In each experiment, the Col-0 and mutant seeds used were from plants from the same batch of harvest.

5.3 | Measurements and statistical analyses

Hypocotyl length and cotyledon area measurements were performed on 4-day-old seedlings using a digital camera (Canon EOS DSLR) and ImageJ software (National Institutes of Health) and analyzed using Excel (Microsoft). Mean values were calculated to determine relative differences between untreated and treated seedlings. To account for the unequal sample sizes across treatments each group was analyzed separately, split either by light (Dc, WLC, Rc, and FRc), or by light (as previous) and mutant line (Col-0, *phyB-9*, and *phyA-211*) with non-parametric tests. For each comparison, a Kruskal–Wallis rank sum test (using `kruskal_test` in *r*) was used to estimate the impact of Azomite concentration (.0, .1, .5, and 1 g/L) under the aforementioned light conditions on hypocotyl length or cotyledon area. Significant effects were further probed by performing a Dunn post hoc test (using `dunn_test` in *r*) on the same model structure, with *p* values adjusted for multiple testing comparisons.

5.4 | Azomite volcanic ash composition

Azomite (A to Z Of Minerals Including Trace Elements, AZOMITE® Mineral Products, Inc.) is surface mined from naturally deposited volcanic ash (Nephi, UT). In composition, Azomite is a lightly weathered DTB, it is classified as a hydrated calcium sodium aluminosilicate and listed as an anticaking agent (United States Code of Federal



Regulations, 21 CFR 582.2729). It is a natural, highly mineralized complex silica ore, with on average over 70 minerals, trace elements, 529.7 ppm of total rare earth elements, including lanthanides (Chemical analysis, AZOMITE® Mineral Products, Inc.). Azomite is a mixture of different substances present in pulverized bits of rock, crystallized minerals, and volcanic glass (Dr. Barry Bickmore, Brigham Young University, *personal communication*); it is produced as a product in different grades from Granulated to Micronized (AZOMITE® Mineral Products, Inc.). For the work presented here, some of the known agriculturally important elements found in Azomite formulations are potassium, phosphorus, calcium, sodium, iron, magnesium, and manganese, along with trace amounts of zinc, copper, molybdenum, and selenium. Based upon multiple sample analysis, the manufacturer provides a guaranteed analysis of (0-0-.2 of N-P-K), along with 1.8% Ca, .5% Mg, and .1% of Cl and Na. Potassium is present as soluble potash (K₂O).

AUTHOR CONTRIBUTIONS

Rajnish Khanna conceived and designed the study. Kent F. McCue and Rajnish Khanna conducted the experiments and obtained seedling images and data with support in plating and measurements from Robert Reed and Alexis Ortiz. Elijah Mehlferber performed statistical analysis and produced graphical figures. Jon Ferrel provided product-specific details in the study design. Kent F. McCue and Rajnish Khanna drafted the manuscript supplemented with technical and editorial comments from all authors.

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CONFLICT OF INTEREST STATEMENT

Rajnish Khanna is the founder of i-Cultiver, an independent company providing consultation and research assistance to food and agricultural industries. All aspects of this study were performed by independent researchers. The authors declare that they have no competing interests. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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