PLANT SILICON INTERACTIONS BETWEEN ORGANISMS AND THE IMPLICATIONS FOR ECOSYSTEMS

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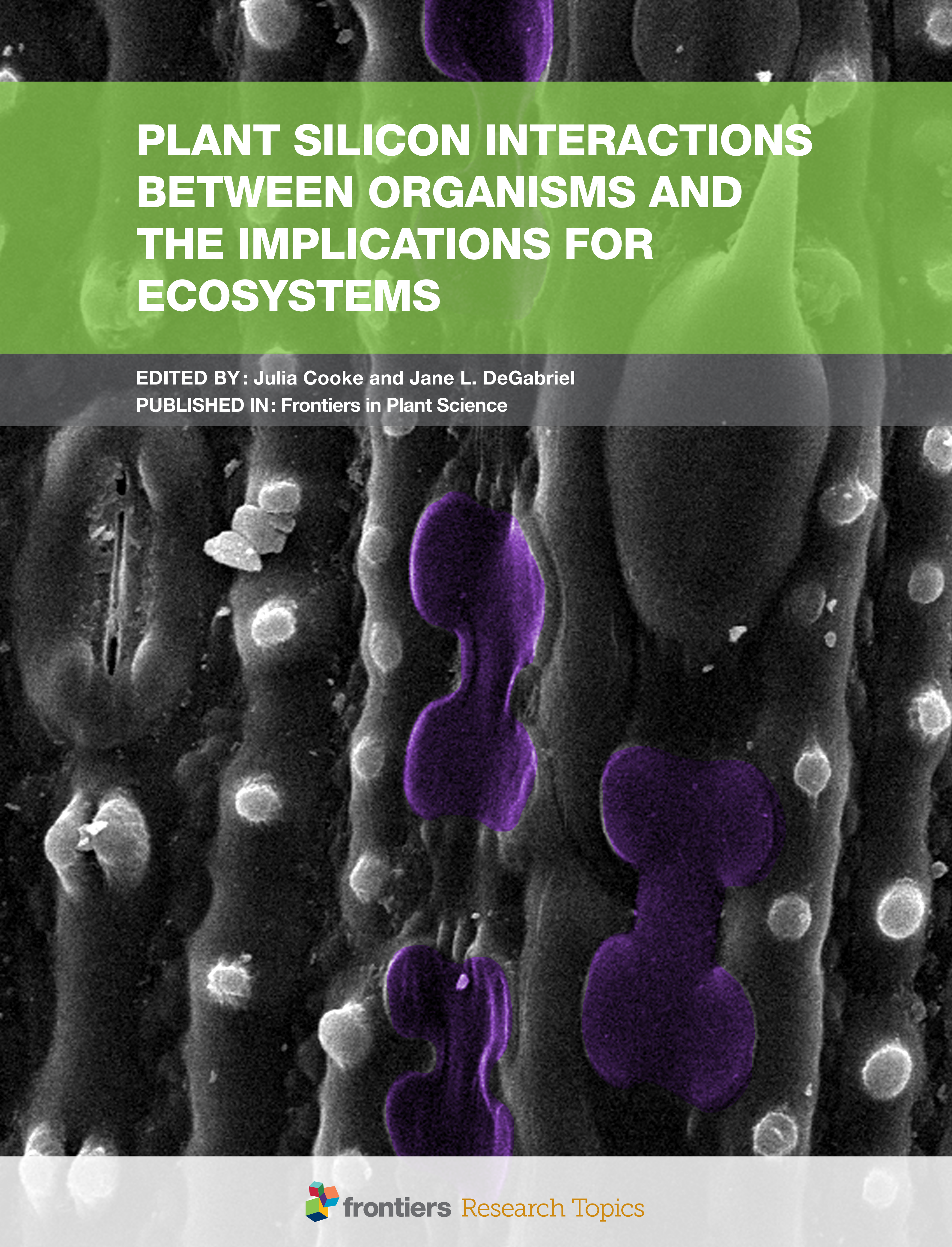

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PLANT SILICON INTERACTIONS BETWEEN ORGANISMS AND THE IMPLICATIONS FOR ECOSYSTEMS

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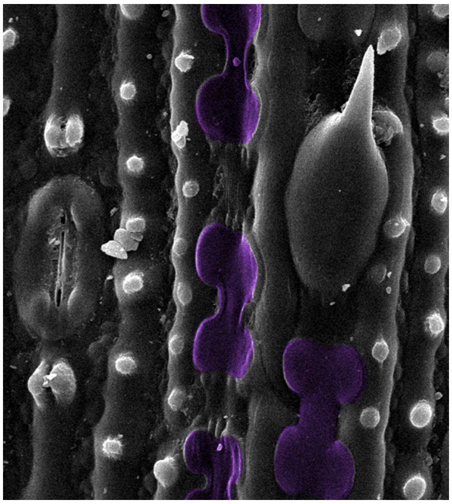

Silica concentration of the Serengeti grass *Themeda triandra* varies according to biotic and abiotic conditions, including water availability. Silica occurs in leaf tissue as solid deposits called phytoliths, including the ~20 µm short cells coloured purple in this SEM image. Figure by K.M. Quigley

In this Frontiers topic, we explore how the functions and fates of plant silicon interact with other organisms and ecosystem processes. By bringing together new data from multiple disciplines and scales, we present a cross-section of novel explorations into how plants use silicon and the implications for agriculture and ecosystems. Key aims in this field are to understand the determinants of plant silicon uptake and cycling, and the benefits that silicon uptake confers on plants, including reducing the impacts of stresses such as herbivory. Current research explores inter-specific interactions, including co-evolutionary relationships between plant silicon and animals, particularly morphological adaptations, behavioural responses and the potential for plant silicon to regulate mammal populations. Another emerging area of research is understanding silicon fluxes in soils and vegetation communities and scaling this up to better understand the global silicon cycle. New methods for measuring plant silicon are contributing to progress in this field. Silicon could help plants mitigate some effects of climate change through alleviation of biotic and abiotic stress and silicon is a component of some carbon sinks. Therefore, understanding the role of plant silicon across ecological, agricultural and biogeochemical disciplines is increasingly important in the context of global environmental change.

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EDITORIAL
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Editorial: Plant Silicon Interactions between Organisms and the Implications for Ecosystems

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Keywords: plant silicon, biogeochemical cycles, herbivore defense, phytoliths, silica

The Editorial on the Research Topic

Recent Advances in the Ecology of Plant Silicon: Novel Insights into Functions, Interactions and Methods for Analysis

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Silicon (Si) is a beneficial, if not essential, plant nutrient (Epstein, 1994). As the second most abundant element in the Earth's crust it has a global cycling budget similar to that of carbon (Conley, 2002). Some ecological roles of Si are characterized (Cooke and Leishman, 2011), but recent technological advances mean unprecedented understanding of functions at multiple scales, and recognition of its importance to global biogeochemical cycling and food security. We present eight original research papers and an opinion article highlighting the novelty and diversity of recent research. New methods, fresh approaches in both applied and fundamental Si research, innovative herbivore defense experiments, ecosystem-scale field measurements, and Si changes under climate change are investigated. The diversity of topics reveals the complexity of plant responses in terms of Si accumulation, distribution, and function, which are contingent on genotype, biotic interactions, and environmental conditions.

High Si-accumulating plant species and families (especially Poaceae) have dominated Si research, including many articles in this research topic. Katz remind us that aside from Poaceae and economically important families such as Fabaceae and Cucurbitaceae, Si has functional roles in low Si-accumulating species. Katz argued that inter-familial variation among taxa is evidence of an ecologically important trait, and that research on low accumulators is likely to facilitate greater understanding of plant Si function.

Regardless of species, efficient methods for quantifying Si concentrations in plants are required. Smis et al. described a new approach to determine Si concentrations using near infrared reflectance spectroscopy (NIRS). They developed calibrations for predicting Si concentrations across diverse plant groups, with improved accuracy when models were restricted to a single species or family. The advantages of NIRS are that it is non-destructive, fast, and cheap, but it relies on robust calibrations from traditional laboratory analyses, such as X-Ray Fluorescence (Reidinger et al., 2012). Further work is required to standardize and improve techniques, but cost and time-effective procedures such as NIRS will surely facilitate ecological advances.

Silicon is added artificially in agriculture to reduce stress and improve production (Ma, 2004; Reynolds et al., 2009), which should be optimized and integrated into management. Keeping et al. examined impacts of Si, N, and water stress on two pests which reduce worldwide sugarcane production. Si addition significantly reduced borer damage, but did not impact the abundance of sucking thrips, perpetuating debate about how Si affects different herbivore types (Reynolds et al., 2009). Contrasting effects of Si on herbivores were found within a single plant, with higher Si resistance in stalks attacked by borers, but lower resistance in leaf spindles eaten by thrips.

Si accumulation can be induced by herbivory, but the trigger for and extent of the induced response is complex (McNaughton and Tarrants, 1983; Massey et al., 2007). Quigley and Anderson found Si induction was dependent on genotype and phylogeny, Si and water availability, grazing type and intensity, with water balance particularly important for some species. They showed consistently lower responses to artificial vs. real herbivory, contingent on damage frequency and intensity, consistent with other papers here (Huitu et al.; Hartley et al.).

Differences in induced Si accumulation and mechanisms of herbivore defense among and within plant species are poorly understood. Hartley et al. examined disparities in plants' ability to alter Si defenses in response to herbivory and Si supply. Species with similar total Si varied in leaf surface abrasiveness, because abrasive grasses had silicified spines, while others deposited silica in short cells. The quantity and distribution of Si was a complex interaction between genotype, Si availability, and herbivore damage. The authors argue that Si allocation to spines impacts palatability, while allocation to short cells may impact digestibility. Thus, there is no single anti-herbivore strategy of Si use by grasses.

Most studies consider Si defenses in isolation, but Huitu et al. examined interactions with another herbivore defense, endophytes (toxin-producing fungi). Tantalizingly, they demonstrated that Si concentrations were consistently ∼16% higher in grasses with endophytes than those without, a similar magnitude to herbivore-induced Si uptake. This result was consistent across grazing levels with no interaction between grazing intensity, endophyte infection, and Si uptake detected. Endophytes could facilitate Si uptake and be beneficial against herbivory, although the higher Si uptake in endophyte-infected plants could also be a response to the intrusion of a foreign body.

Plant Si accumulation is complex, with aquaporins facilitating uptake in some species (Ma and Yamaji, 2015). Most studies have explored uptake using controlled experiments, but empirical data is needed to understand natural plasticity. At an ecosystem level, Carey and Fulweiler showed substantial natural variation in Si accumulation in *Spartina*, using new and published data. A conceptual model suggested that uptake is more plastic than previously recognized, driven by environmental factors and genetic origin. This challenges attributing a single Si accumulation value to a species and highlights that we lack a definitive method to characterize accumulation capacity.

Si weathers from primary silicates and cycles between plants and soils, eventually leaching into rivers and oceans. Cycling rate regulation is a hot topic and Li et al. investigated the dissolution potential of plant silica bodies (phytoliths) originating from different parts of rice plants. Phytoliths from leaves and sheaths were likely to contribute to faster recycling rates compared to grains and stems, linked to higher Si/Al and Si/Fe ratios. Genotype, morphology, and hydration rate also affected Si cycling. Consequently, variation in Si accumulation/deposition associated with herbivory, Si availability, and water balance described by others (Carey and Fulweiler; Quigley and Anderson; Hartley et al.) could have implications for Si cycling.

How plants Si accumulation and use might respond to climate change is unknown. Fulweiler et al. provide a first insight using data from a free-air CO_2 enrichment experiment. They showed little change in foliar Si concentration under elevated atmospheric CO_2. However, due to increased primary production, Carey and Fulweiler suggested that elevated atmospheric CO_2 could significantly increase the Si pumping capacity of the vegetation in this system by up to 26%, with implications for C sequestration and downstream systems. Changes in transpiration rates under changing CO_2 could also be a factor determining Si uptake in future climates.

Silicon concentration varies more than most elements among and within species (Epstein, 1999). This research topic presents novel advances in understanding the relationship between this variation and plant Si use. Factors determining accumulation are complex and often plastic, making further understanding an intriguing and promising challenge. An immediate challenge is understanding how global environmental change is likely to impact on Si in ecology and agriculture and the implications for ecosystem services and food security.

AUTHOR CONTRIBUTIONS

JC and JLD are joint first authors of this paper, having equally contributed to the development of this research topic and its editorial.

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Beyond grasses: the potential benefits of studying silicon accumulation in non-grass species

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INTRODUCTION

Silicification in angiosperms is a phenomenon that has attracted increasing attention in recent years. It is now widely acknowledged that silicification has many benefits to angiosperms (Richmond and Sussman, 2003; Ma and Yamaji, 2008; Epstein, 2009; Guntzer et al., 2012), and that it probably plays appreciable roles at the ecosystem and landscape levels as well (Cooke and Leishman, 2011; Reynolds et al., 2012; Schoelynck et al., 2014). High silica accumulating plant species are considered a major pool in the silicon cycle, affecting silicon fluxes and turnover rates (Conley, 2002; Falkowski et al., 2004; Derry et al., 2005; Sommer et al., 2006; Li et al., 2011; Carey and Fulweiler, 2012; Struyf and Conley, 2012; Vandevenne et al., 2013; Schoelynck et al., 2014). Indeed, recent studies have demonstrated the significant roles of plant silicification on the silicon cycle in grasslands (e.g., Melzer et al., 2012), freshwater and tidal ecosystems (e.g., Jacobs et al., 2013; Schoelynck et al., 2014) and forests (e.g., Farmer, 2005; Farmer et al., 2005; Cornelis et al., 2011). Furthermore, since silicate weathering is a CO_2-consuming process, the effects of silica uptake and accumulation by plants on the silicon cycle may also influence the global carbon cycle (Street-Perrott and Barker, 2008).

Silica contents vary greatly among the angiosperms, with high concentrations occurring most commonly in the Poaceae (hereafter referred to as grasses) and other related monocotyledonous commelinid families (Hodson et al., 2005; Piperno, 2006) and aquatic macrophytes (Schoelynck et al., 2012). Due to the relatively high silica concentrations in grasses and to their high economic and ecological importance, silicification has been studied more frequently and more intensely in this family than in any other plant family. However, a better understanding of silicification in non-grass taxa can promote our understanding of the function and importance of silicon for plants. Unfortunately, studies of non-grass taxa are not only rarer, but also usually focus on a small number of taxa, mostly families and species that are silica-rich (e.g., Cucurbitaceae: Rogalla and Römheld, 2002), economically important (e.g., Fabaceae: Shen et al., 2010), or serve as model plant species (e.g., *Arabidopsis*: Fauteux et al., 2006), which are unlikely to be a fair representation of non-grass taxa. This means there is a significant gap in our knowledge of silicon processes, patterns and roles, given the fact that non-grass taxa, such as forest tree, are a major component of the silicon cycle (Farmer, 2005; Farmer et al., 2005; Cornelis et al., 2011). I shall therefore briefly discuss the variability of silicification among non-grass taxa, and how it can be used to promote a better and broader understanding of this phenomenon.

LOW SILICA CONCENTRATIONS ARE NOT NECESSARILY "INSIGNIFICANT"

Recent studies on non-grass taxa have revealed that the dichotomous view of angiosperm taxa as being either high silica accumulators (e.g., grasses, other commelinids, and Cucurbitaceae) or low silica accumulators (i.e., most non-commelinid angiosperms) is flawed. First, some dicotyledonous species (other than those belonging to the Cucurbitaceae; e.g., *Abies pectinata*, *Cajanus cajan*, *Fagus sylvatica*, and *Helianthus annuus*) have silica concentrations which are as high or almost as high as those found in grasses (e.g., Hodson et al., 2005; Piperno, 2006). Second, there appear to be fundamental differences in the type, magnitude, patterns and functions of silicification between the high silica accumulating grasses and the low silica accumulating non-grass taxa. In grasses, it is generally accepted that silicification increases under higher silicon and water availability (reviewed in Katz et al., 2013) or if plants are exposed to prolonged herbivory (e.g., Soininen et al., 2013). In comparison, the effects of silicon and water availability on silicon accumulation in non-grass taxa are less clear. Cooke and Leishman (2012) found that silicification in non-grass species is probably less dependent on soil silica availability compared to silicification in grasses. Euliss et al. (2005) and Katz et al. (2013) found that the effects of water availability on silicification in non-grass species are weaker than in grasses growing under otherwise similar conditions. Herbivory seems to promote silicification more commonly in grasses compared to southwest Asian Asteraceae species, and it is thus also probable that silica plays less of a defensive role in Asteraceae species (Katz et al., 2014). Yet, even in plant species with very low silica concentrations, silica may play substantial roles, including reducing aluminum toxicity (Hodson and Evans, 1995; Britez et al., 2002; Khandekar and Leisner, 2011) and defending plants from pathogens (Fauteux et al., 2006; Zellner et al., 2011). Thus, low silica accumulation is not indicative of low silica function, and therefore silica function is not restricted to high silica grass taxa.

DIFFERENT FAMILIES, DIFFERENT VARIABILITY

There is both inter- and intra-familial variation in silica accumulation in plants. The grass family (approximately 10,000 species) and other commelinid families consist of high silica accumulators in which silicification patterns and roles are highly consistent. The effects of water availability (reviewed in Katz et al., 2013) and herbivory (Soininen et al., 2013) on silicification, as well as the multiple roles silica plays in grasses (Richmond and Sussman, 2003; Epstein, 2009; Cooke and Leishman, 2011; Guntzer et al., 2012), serve as good examples. The Orchidaceae (22,000-26,000 species) is a non-commelinid monocotyledonous family that is the most closely related to the commelinids (Bremer, 2000). The more ancient orchid subfamilies, Apostasioideaea and Cypripedioideae, are characterized by high silica contents, while members of the more derived Vanilloideae and Orchidoideae, and some Epidendrioideae species, have very low silica concentrations (Prychid et al., 2004). The loss of this trait, most notably in parallel to the transition from epiphytism to land-dwelling, is an evolutionary change that deserves more attention.

The Cucurbitaceae (1000 species) is a dicotyledonous family of high silica accumulators, but given there are dissimilarities in silicification mechanisms, patterns and roles between the two families it is likely that high levels of silicon accumulation evolved independently in this family (e.g., Mitani and Ma, 2005). The Asteraceae and Fabaceae, two of the largest angiosperm families (23,000 and 20,000 species, respectively) also demonstrate wide ranges of silica concentrations (Hodson et al., 2005; Piperno, 2006; Katz et al., 2013, 2014). At least in the case of the Asteraceae, it is now known that silicification patterns are probably poorly dependent on intra-familial phylogeny and soil silica concentrations (Katz et al., 2013, 2014). In other angiosperm families, such as the Rubiaceae (13,000 species), most studied species were shown to have very low silica concentrations (Piperno, 2006). Thus, there seem to be interesting patterns of silicification in families other than Poaceae which are likely to demonstrate variation in silica function and importance.

CONCLUDING REMARKS

It is clear that some groups, such as the grasses, commelinids and some aquatic macrophytes (Schoelynck et al., 2012), evolved the ability to accumulate silicon as herbivore defenses and a mechanism of alleviating abiotic stresses (e.g., Strömberg, 2011). Yet, these taxa represent only a part of the overall diversity of silicification among angiosperms. The inter-familial variability among non-grass taxa suggests that silicification is an ecologically-important trait in some of these taxa as well, especially in taxa which show silicification patterns resembling those found in grasses. Further studies of silica contents, silicification patterns and roles of silica in non-grass taxa are likely to elucidate physiological, ecological and evolutionary processes underlying the inter- and intra-familial variability in silica accumulation. This has the potential to increase our understanding of silicification and its significance in the biology and ecology of many species at a faster rate than through the study of Poaceae alone.

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Determination of plant silicon content with near infrared reflectance spectroscopy

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Silicon (Si) is one of the most common elements in the earth bedrock, and its continental cycle is strongly biologically controlled. Yet, research on the biogeochemical cycle of Si in ecosystems is hampered by the time and cost associated with the currently used chemical analysis methods. Here, we assessed the suitability of Near Infrared Reflectance Spectroscopy (NIRS) for measuring Si content in plant tissues. NIR spectra depend on the characteristics of the present bonds between H and N, C and O, which can be calibrated against concentrations of various compounds. Because Si in plants always occurs as hydrated condensates of orthosilicic acid ($Si(OH)_4$), linked to organic biomolecules, we hypothesized that NIRS is suitable for measuring Si content in plants across a range of plant species. We based our testing on 442 samples of 29 plant species belonging to a range of growth forms. We calibrated the NIRS method against a well-established plant Si analysis method by using partial least-squares regression. Si concentrations ranged from detection limit (0.24 ppmSi) to 7.8% Si on dry weight and were well predicted by NIRS. The model fit with validation data was good across all plant species ($n = 141$, $R^2 = 0.90$, RMSEP $= 0.24$), but improved when only graminoids were modeled ($n = 66$, $R^2 = 0.95$, RMSEP $= 0.10$). A species specific model for the grass *Deschampsia cespitosa* showed even slightly better results than the model for all graminoids ($n = 16$, $R^2 = 0.93$, RMSEP $= 0.015$). We show for the first time that NIRS is applicable for determining plant Si concentration across a range of plant species and growth forms, and represents a time- and cost-effective alternative to the chemical Si analysis methods. As NIRS can be applied concurrently to a range of plant organic constituents, it opens up unprecedented research possibilities for studying interrelations between Si and other plant compounds in vegetation, and for addressing the role of Si in ecosystems across a range of Si research domains.

Keywords: NIRS, plant silica concentration, calibration, Fennoscandia, ecosystem research, graminoids, *Deschampsia cespitosa*

INTRODUCTION

Silicon (Si) is widely present in the plant kingdom. In most plant species, plant Si constitutes ~0.1 up to 2% of Si by plant dry weights (Epstein, 1994; Hodson et al., 2005). Remarkably high average concentrations in some plant groups such as horsetails (~3%Si by dry weight), and grasses (~1.5%Si by dry weight; Hodson et al., 2005) suggest that Si can have an essential role in plants. Si supports plant growth, improves the plant structural strength, viability, reproduction and resistance against biotic (e.g., parasitism, pests, herbivory) and abiotic (e.g., metal toxicity, salinity, drought) stresses, as well as increases the efficiency of light interception (Epstein, 1999; Ma et al., 2001). Hence there are compelling reasons to study Si in plants. However, the importance of plant Si reaches beyond the plant kingdom as the Si-assimilation of plants is coupled to the global biogeochemical Si and C cycling (Street-Perrott and Barker, 2008). Plants significantly increase mineral silicate weathering and the coupled CO_2 drawdown (Kelly et al., 1998; Moulton et al., 2000). Plant Si uptake functions as a "terrestrial Si filter" (Struyf and Conley, 2012), controlling the continuous delivery of Si to rivers that connect the continental Si cycle to the coastal and oceanic Si cycle (Tréguer and De La Rocha, 2013). As such, plant biosilicification is tightly linked to aquatic eutrophication problems (Cloern, 2001), and the strength of the oceanic carbon sequestration (Ragueneau et al., 2006), making the study of plant Si in terrestrial ecosystems instrumental to climate change studies.

Despite the increasing interest, the quantification of biogenic silica (bSi) in terrestrial ecosystems is hampered by the time-intensive and relatively expensive chemical analysis methods for bSi. In most studies, the total plant Si content, further indicated as plant bSi, is analyzed by chemical digestion followed by a colorimetric or a spectrophotometric analysis method (e.g., Massey et al., 2007; Saccone et al., 2007; Vandevenne et al., 2012; Carey and Fulweiler, 2013; Meunier et al., 2013), or by wet or dry ashing followed by gravimetric analysis (e.g., Carnelli et al., 2001; Ma et al., 2001; Blecker et al., 2006). These methods are destructive, time-intensive and require chemical pre-treatment. Especially in large-scale studies and monitoring programs, more efficient methods are warranted for a better understanding of both the role of Si in ecosystems as well as the biosilicification at the scale of entire ecosystems.

In this study, we address the applicability of a comparable non-destructive method, Near Infrared Reflectance Spectroscopy (NIRS), for the analysis of Si in plants. NIRS has been used intensively in agricultural and industrial research (e.g., Shenk and Westerhaus, 1994), and is receiving increasing interest in ecology after numerous successful ecological applications have been demonstrated, mostly for the determination of plant primary and secondary organic compounds and parameters related to the food quality of plants (e.g., Stolter et al., 2006; Chodak, 2008). Plant material absorbs near infrared radiation (NIR) where there is presence of chemical bonds between hydrogen (H) and other atoms (e.g., C–H, N–H, O–H; Kaye, 1954). Si occurs in plants in and between plant cells as amorphous opal silica structures called phytoliths or phytogenic Si (Sangster and Hodson, 1986; Piperno, 1988). These structures are always composed of hydrated condensates of orthosilicic acid ($Si(OH)_4$), containing mostly Si-O-Si bonds, and some hydroxyl groups ($SiO_2.nH_2O$; Currie and Perry, 2007). In addition, these silica structures show strong interactions with several plant biomolecules (e.g., cellulose; Perry and Lu, 1992). Although Si never directly binds to H, the hydrated silica structures probably interact with their plant organic environment through hydroxyl groups (Zhang et al., 2013) and are expected to result in a specific NIR absorption signature of plant bSi. This suggests a possibility to apply NIRS as analysis method of plant Si.

The reasons to apply NIRS are manifold: samples need minor pre-treatment without the use of chemicals, the analysis itself is rapid, non-destructive, allows repeated measurements, and it provides information about many plant parameters from a single sample (Foley et al., 1998). Recently, X-ray fluorescence (XRF) spectroscopy was presented as a more rapid and accurate alternative to digestion-based chemical techniques (Reidinger et al., 2012). Although XRF spectroscopy has shown to have the same advantages regarding analysis time, analysis cost, and sample consumption, XRF spectroscopy cannot be used for the simultaneous analysis of numerous plant organic compounds for which NIRS have been shown to be successful and which are often of great interest in ecological research (e.g., Schaller et al., 2012).

The applicability of NIRS for Si analysis has been tested using agricultural (alfalfa hay, rice straw) and bio-industrial products (sugar mill mud and ash; Halgerson et al., 2004; Jin and Chen, 2007; Ostatek-Boczynski et al., 2013). Although they all suggested a potential applicability of NIRS, none of these studies showed sufficiently strong calibrations for direct application on plant material. Jin and Chen (2007) used the insoluble ash method for their reference values, although this method was only recommended if the content of indigestible mineral residues is sought (Van Soest and Jones, 1968). Halgerson et al. (2004) used a calibration data set with a very narrow concentration range around and average Si concentration of 0.02%, and Ostatek-Boczynski et al. (2013) only tested NIRS on industrially processed plant material (sugar mill by-products). For ecological applications, the widespread nature of Si in the plant kingdom calls for testing the general applicability of NIRS, i.e., a method that can be applied both to the wide range of plant taxa and over the typical concentration range in which bSi is found in nature. With support from these moderately successful pioneer studies, we hypothesize in this study that NIRS may be applied for measuring the Si content of plants.

MATERIALS AND METHODS

PLANT SAMPLING

The study was performed in the alpine parts of the northern, subarctic zone of Norway (Finnmark). Plant species were sampled from eight river valleys, extending the temperature gradient of the region. The sampled plant species (**Table 1**) are typical for the riparian zone, dominated by willows, forbs and grasses, and for the surrounding dry heath tundra, dominated by dwarf shrubs. Each sample consisted of several plant individuals from the same location, and habitat, with a maximum distance of 25 m from each other. Plant individuals were always sampled by harvesting the green non-woody plant parts; the whole plant in the case of grasses, forbs and horsetails, and the leaves in the case of shrubs (**Table 1**). Plant material was cut some centimeters above the soil level and sampled plant material was always visually controlled to be free from soil particles. Samples of 29 plant species, representing different growth forms, were collected making a total of 442 plant samples (**Table 1**). In order to cover plant and Si structural variations over several plant development stages, we sampled plant species at the beginning (June), during (July) and at the end of the growing season (August).

THE REFERENCE CHEMICAL ANALYSIS METHOD

Plant samples were oven dried for 3 days at 75°C. We chose not to wash the plant samples as sampling routines excluded the presence of soil particles and the ecological context (high precipitation, dense vegetation, long distance to roads) restricted the possibilities for aeolean dust/soil transport. Subsequently, samples were pulverized using a ball mill (Mixer Mill, MM301; Retsch GmbH and Co. Haan, Germany) in order to get very fine and homogeneous plant material. We applied a chemical digestion followed by a colorimetric analysis, as the gravimetric approach has been proven to show a poor reproducibility (Herbauts et al., 1994). Plant bSi was extracted by the wet alkaline (0.1 M Na_2CO_3) extraction, a technique recently confirmed to be suitable for bSi analysis of plant material (Meunier et al., 2013). About 30 mg of pulverized plant material was incubated for 4 h in 0.1 M Na_2CO_3 at 80°C (DeMaster, 1991) and then 10 ml of the extract was filtered (Chromafil® A-45/25, pore size of 0.45 μm). After extraction, samples were stored in a dark room at 3°C and were analyzed colorimetrically for extracted dissolved Si (DSi) within a maximum of 2 weeks, using a Continuous Flow Analyser (CFA; SKALAR SA 1500, Smith and Milne, 1981). Blank extractions were subtracted to account for Si release from recipients and chemicals. The error of the entire analysis procedure was measured as the (reproducibility) standard deviation of the analysis of

Table 1 | Overview of plant species, plant part, sample size (*n*), and the plant silicon concentration range, average value and the coefficient of variation of the plant silicon concentration for all studied growth forms with species alphabetically ordered within each growth form.

Growth form ***Plant species***	**Plant part**	**Total**	**Silicon concentration (% on dry weight)**		
			Range (min–max)	**Average value**	**Coefficient of variation (%)**
Graminoids		**265**	**0.072–9.991**	**1.054**	**195**
Agrostis tenuis	WP	7	0.396–3.868	1.351	92
Alopecurus pratensis	WP	2	0.201–0.778	0.489	83
Anthoxanthum nipponicum	WP	21	0.177–1.287	0.541	45
Avenella flexuosa	WP	56	0.084–1.066	0.419	54
Calamagrostis sp.	WP	23	0.290–3.060	1.417	48
Carex bigelowii	WP	35	0.073–2.912	0.690	86
Deschampsia cespitosa	WP	75	0.341–9.991	1.575	71
Festuca ovina	WP	3	0.424–1.142	0.728	51
Nardus stricta	WP	30	0.555–3.620	1.728	35
Phleum alpinum	WP	13	0.114–0.411	0.250	37
Forbs		**99**	**0.002–2.875**	**0.203**	**232**
Alchemilla sp.	WP	40	0.003–2.875	0.454	147
Bistorta vivipara	WP	11	0.002–0.021	0.009	71
Chamaepericlenum suecicum	WP	2	0.007–0.007	0.007	4
Comarum palustre	WP	1	–	0.068	–
Geranium sylvaticum	WP	9	0.003–0.025	0.012	58
Ranunculus sp.	WP	4	0.036–0.634	0.196	149
Rubus chamaemorus	WP	2	0.056–0.072	0.064	18
Rumex acetosa	WP	7	0.009–0.037	0.019	57
Solidago virgaurea	WP	10	0.018–0.143	0.058	83
Trientalis europaea	WP	1	–	0.006	–
Trollius europaeus	WP	6	0.002–0.034	0.016	75
Viola sp.	WP	6	0.006–0.040	0.022	62
Shrubs and Dwarf shrubs		**50**	**0.000–0.053**	**0.010**	**100**
Betula nana/pubescens	L	5	0.000–0.010	0.004	86
Empetrum nigrum	L	12	0.003–0.053	0.015	87
Salix sp.	L	28	0.001–0.039	0.011	86
Vaccinium myrtillus	L	4	0.003–0.011	0.006	59
Vaccinium vitis-ideae	L	1	–	0.001	–
Horsetails		**26**	**0.038–7.797**	**2.909**	**71**
Equisetum sp.	WP	26	0.038–7.797	2.909	71

WP, whole plant; L, leaves.

12 subsamples of a single pulverized plant sample (*Deschampsia cespitosa*).

ANALYSIS WITH NIRS

Remaining pulverized plant samples were made into tablets (Ø16 mm, >1 mm thick) by applying 6 tons of pressure with a hydraulic press, in order to obtain a homogeneous flat surface. A homogeneous flat surface reduces random light scatter of loose powder, thus reducing random variation in spectral signatures. Before NIRS analyses, the plant tablets were oven dried at 50°C for 2 h in order to remove the remaining water film on the hydrophilic plant material. The dry samples were then cooled in a desiccator until scanning by NIRS. The NIRS-spectra, measured as the logarithm of the inversed reflectance (log(1/R)), were recorded with monochromatic radiation at 1.4 nm intervals from 350 to 1050 nm, and at 2 nm intervals from 1000 to 2500 nm, using a FieldSpec 3 (ASD Inc., Boulder, Colorado, USA) and were registered at 1 nm interpolated intervals. Each sample spectrum was recorded as the average of three replicate scans. NIRS scanning was performed at room temperature during one single winter day, which most likely resulted in a stable room temperature as there was no sunlight to warm up the room.

CALIBRATION AND VALIDATION

Three nested calibration sets were created; one on the total sample size, representing all studied plant species ($n = 442$); one for a subset containing all graminoids ($n = 264$), which are, as active Si accumulators, most often studied in plant-Si studies, and finally, in order to test the performance of NIRS when applied to one single plant species, one calibration for a subset containing all samples of the grass species *Deschampsia cespitosa* ($n = 75$). All data sets were treated similarly: in order to get a similar spectral diversity within the calibration and validation dataset, calibration and validation samples were selected using the Kennard-Stone algorithm (Kennard and Stone, 1969) included in the "prospectr" package 0.1.3 (Stevens and Ramirez-Lopez, 2014) in R 3.0.1 (R Development Core Team, 2013). Spectral outliers were identified by means of Mahalanobis distances (75% quantile as cutoff point; Mahalanobis, 1936; De Maesschalck et al., 2000) and removed in a single operation. Before starting the calibration, the wavelength regions where the NIRS sensors overlapped (350–380 nm, 760–840 nm, 1700–1800 nm and 2450–2500 nm) were removed to avoid false correlations from instrumental noise.

The statistical procedure for the calibration, using partial least-squares regression (Martens and Næs, 1993), in the pls-package 2.4–3 (Mevik et al., 2013) in R 3.1.0. Different transformations on the spectral data set were considered for the calibrations: scaling and/or centering, smoothing before applying derivatives, and the use of first (1D) and second order derivatives (2D) by using the earlier mentioned "prospectr" package (Stevens and Ramirez-Lopez, 2014) in R.

The most parsimonious calibration model was chosen based on evaluation of two calibration statistics: a high coefficient of multiple determination (R^2), and the lowest root mean square error of calibration (RMSEC) for a given number of selected model components k. Overfitting was avoided by selecting the combination of model parameters k showing the lowest standard error of cross-validation (SECV; Mark and Workman, 1991). Finally, the chosen calibration model was used to predict the Si concentrations of the validation sample sets. The R^2, the RMSEP ("root mean square error of prediction," i.e., the mean error rate between predicted and real values), the bias (i.e., the systematic error of the linear regression), and the intercept and slope of the linear fit of the predictions were used to assess the applicability of the developed NIRS calibration models. We tested the quality of all calibration models with and without the visible region (380–720 nm). Chlorophyll has a strong signature in the visible light spectra, and seasonal variation in chlorophyll content could consequently be confounded with variation in bSi content. As the models with visible light were consistently of lower quality, we only present models without the visible light.

RESULTS

PLANT SI CONCENTRATIONS

We found plant Si concentrations to range from the detection limit (<0.024%Si) to 10.0% of Si by dried plant weight. The average error of the wet chemical analysis method, measured as the reproducibility standard deviation of the repeated measurement of a *Deschampsia cespitosa* sample with average concentration of 0.34%Si, was 0.022% of Si by dry weight, which corresponds to an average relative error of 2.6%. Across all species, plant Si concentration showed large variation with a coefficient of variation of 69%. However, the concentrations of Si were low (<0.1%Si) for almost all shrubs, dwarf shrubs and forbs, while most graminoids and horsetails showed average Si concentrations higher than 0.5% (**Table 1**). The selection of the calibration and validation datasets based on the spectral characteristics of the samples resulted in comparable Si concentration frequency distributions of the calibration and validation datasets for the three separate models (**Figure 1**).

THE NIRS CALIBRATION MODELS

The best calibration model for Si across all plant species was based on the second order derivatives (2D) of the smoothed (17 wavelength intervals) NIR spectra with 23 components, which showed highest loading values at 1050, 1900, and 2300 nm (**Figure 2**). The best calibration model for graminoids was based on the multiplicative-scatter corrected spectra, and 33 components. Highest loading values were found for wavelengths around 1900 and 2300 nm (**Figure 3**). Finally, the best calibration model for the grass species *Deschampsia cespitosa* was based on the centered, second order derivative spectra with 19 components, with highest loading values for the same wavelengths as the calibration model including all plant species (**Figure 4**).

Our calibration model including 442 samples from 29 different plant species showed a RMSEP of 0.24% of Si by dry weight (**Table 2**, **Figure 5**), corresponding to a relative error of 28% for the average measured Si concentration. Our separate calibration model for graminoids showed a RMSEP of 0.10% of Si by dry weight, corresponding to an average relative error of only 8.3% (**Table 2**, **Figure 6**). Narrowing the graminoid dataset to the most abundant grass species, *Deschampsia cespitosa*, increased the calibration model accuracy; a RMSEP of 0.023% of Si by dry weight, corresponding to a relative error of 1.3% (**Table 2**, **Figure 7**). The bias was also highest for the model including all plant species, whereas it was lowest and similar for the graminoids and *Deschampsia cespitosa* (**Table 2**). For the models including all plant species and the graminoids, the slope and intercept of the validation regression lines were close to 0 and 1 respectively, whereas the *Deschampsia cespitosa* model showed a slope close to one, and an intercept which differed more from 0 (**Table 2**). The R^2 values of the validation regression were all equal to or higher than 0.90 and showed a similar pattern as the bias, with

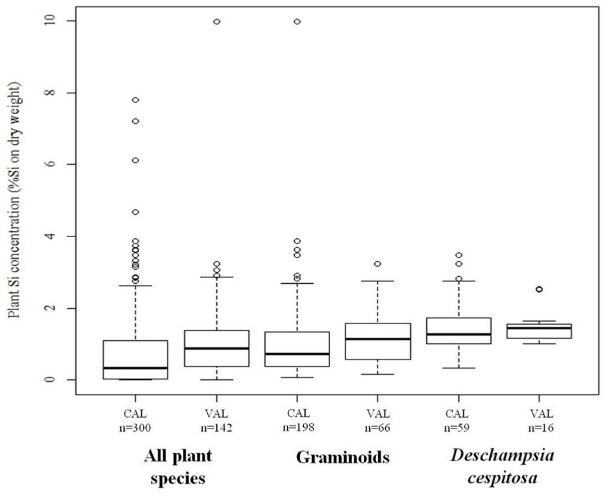

FIGURE 1 | Statistical summary (minimum, first quartile, median, third quartile, maximum value which is not an outlier, and outliers) of the plant silicon (Si) concentrations, measured by chemical analysis, in the calibration (CAL) and validation (VAL) dataset of the "all species" model, the "graminoids" model, and the "*Deschampsia cespitosa*" model.

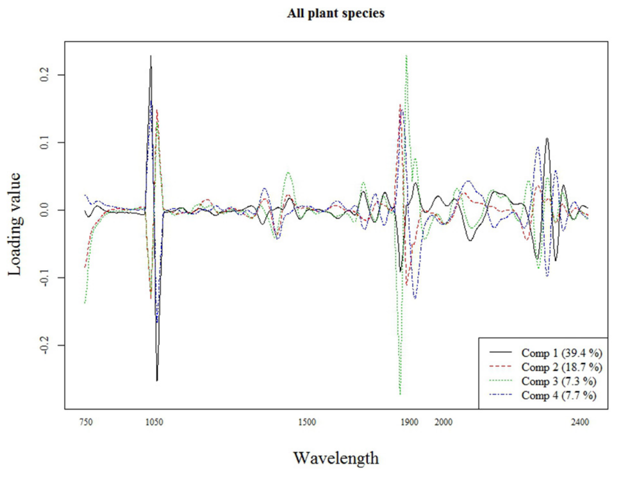

FIGURE 2 | Loading plot for the most important components of the calibration model for all plant species.

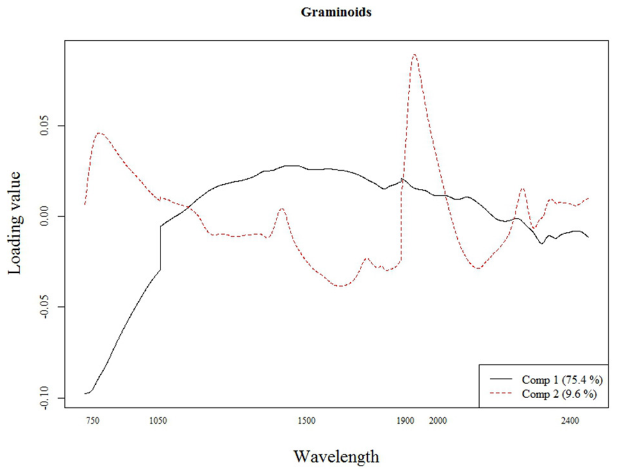

FIGURE 3 | Loading plot for the most important components of the calibration model for the graminoids.

the highest value for the graminoids model (0.95), the lowest for the model including all plant species (0.90) and an intermediate value for the *Deschampsia cespitosa* model (0.93; **Table 2**, **Figures 5–7**).

DISCUSSION

THE QUALITY OF THE NIRS CALIBRATION MODEL

Our results show that the Si content of plants can successfully be measured by NIRS across a large range of plant species. All three calibration models showed a high coefficient of determination ($R^2 \geq 0.9$). The calibration model for the grass *Deschampsia cespitosa* showed the best overall performance with the lowest root mean square error in the validation (RMSEP), and a slope closest to 1 (**Table 2**). Taking into account that the average error of the wet chemical extraction method followed by a colorimetric analysis is 0.022% of Si by dry weight, using NIRS for graminoids increases the average error with 0.078% of Si by dry weight, which is acceptable when working with plants accumulating Si (Si > 0.5%Si by dry weight; Raven, 2003). For the *Deschampsia cespitosa* model, the average error became slightly lower compared to the reference chemical analysis method (0.015%Si vs. 0.022%Si). Also, a further specification of the calibration model from all plant species to the plant group level and finally to a single plant species increased the accuracy. Prudence is called for when using the calibration model across all plant species in studies that require very high accuracy. Although the model containing all plant species showed a high R^2, and an intercept and slope close to 0 and 1, respectively, it was characterized by a RMSEP of 0.24% of Si by dry weight, which is rather high, even for plant species actively accumulating Si.

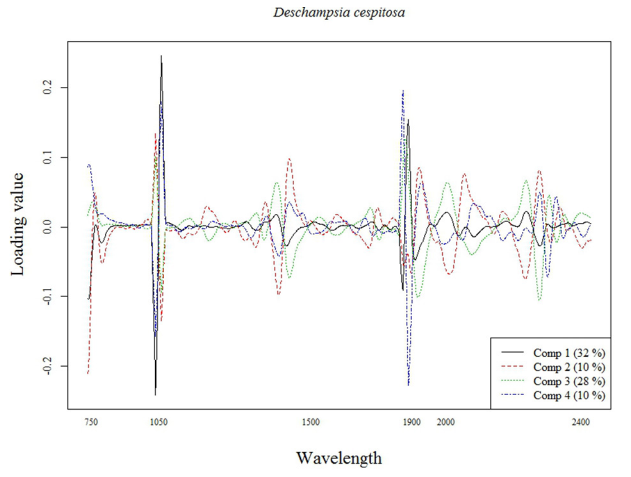

FIGURE 4 | Loading plot for the most important components of the calibration model for *Deschampsia cespitosa*.

Our three calibration models showed remarkably better validation statistics compared to earlier studies on the application of NIRS of plant material (Halgerson et al., 2004; Jin and Chen, 2007; Ostatek-Boczynski et al., 2013), and is probably a result of (1) the use of a well-proven and widely used reference method for plant Si analysis (Meunier et al., 2013), and (2) a calibration dataset which consists of plant species representing almost the entire range of natural plant Si concentrations (Hodson et al., 2005). We also hypothesize that the specific pre-treatment of the plant samples before NIRS scanning, where we pressed ground plant powder into a tablet, may have increased the quality of the calibration. The ball-mill produced very fine powder, resulting in a flat tablet surface which may have reduced both light scattering from powder and original structural differences between plant species. However, as we were not able to measure the fineness, particle size distribution and homogeneity of the ground plant samples, this issue remains subject for further research and the quality of the NIRS calibration could potentially be increased by using a cyclotech mill instead of the ball mill procedure. The cyclotech mill grinding method ensures a narrow particle size distribution, which has been proven to strongly influence the NIR spectrum (Casler and Shenk, 1985).

Table 2 | Calibration and validation statistics (R^2: coefficient of determination; RMSEC: root mean square error of calibration; RMSEP: root mean square error of prediction) of the Partial Least Squares regression models on NIR spectra for the analysis of plant Si concentration in three separate models.

Calibration model	Model selection: calibration				Model quality: validation					
	No. of components (k)	*n*	R^2	RMSEC	*n*	R^2	RMSEP	Bias	Intercept	Slope
All plant species	23	300	0.88	0.3327	141	0.90	0.2379	−0.054	−0.0009	0.95
Graminoids	33	198	0.91	0.2076	66	0.95	0.1021	−0.032	0.0070	0.97
Deschampsia cespitosa	19	59	0.95	0.1357	16	0.93	0.0150	−0.035	−0.0660	1.02

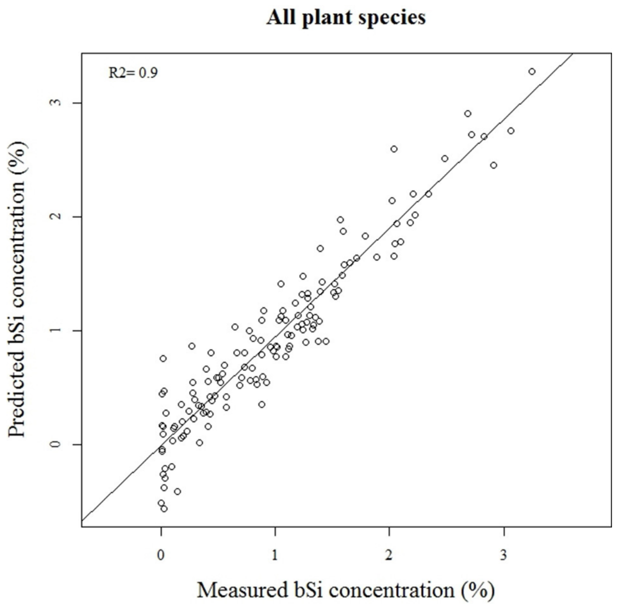

FIGURE 5 | Validation plot of the NIRS calibration model based on samples of all the plant species.

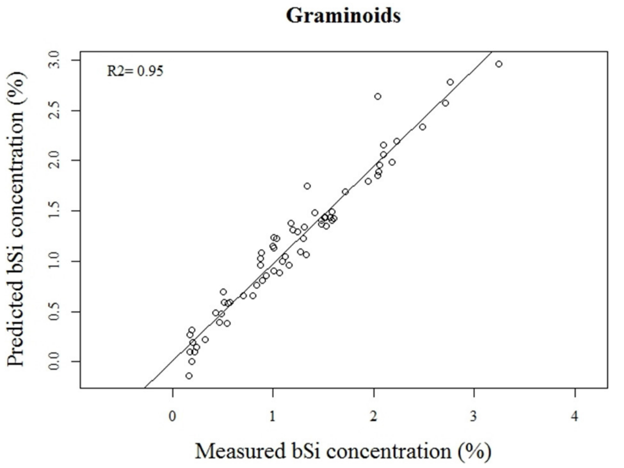

FIGURE 6 | Validation plot of the NIRS calibration model based on samples of graminoids.

NIRS CALIBRATION AND PLANT SI BIOCHEMISTRY

Our success in measuring Si with NIRS across several plant lineages, representing different plant phenological stages and a range of ecological contexts, may also be due to the single type of Si structure in plants. Whereas the often transient and species-specific character of associations between minerals and organic or hydrated molecules (Clark et al., 1987) may limit the applicability of NIRS for mineral analysis to a well-defined population (plant species, sampling time; Foley et al., 1998), Si in plants is only known to occur as a hydrated condensate of orthosilicic acid ($Si(OH)_4$). However, narrowing the calibration dataset to graminoids and finally to *Deschampsia cespitosa* resulted in a clear increase in accuracy (lower RMSEP) and a regression slope close to one in the case of *Deschampsia cespitosa*. We suggest that this increase in calibration model accuracy is related to plant group/species dependent locations of bSi formation. Cell-silicification shows clear phylogenetic differences. In monocotyledons, silica is more often deposited in the cell lumen of "silica cells," and as "silica bodies" on cells, while dicotyledons more often show silica deposits within or underneath the cell wall (Piperno, 1988). In addition, plant lineages differ in the type of tissues which are silicified; in grasses, Si is preferentially deposited as a layer beneath the cuticle (Prychid et al., 2004), the Pteridaceae (ferns) and Equisetaceae (horsetails) mostly show silicification within and at epidermal cells (Kaufman et al., 1971; Sundue, 2009), while orchids (Orchidaceae) are characterized by silica bodies in the sclerenchyma (Møller and Rasmussen, 1984). Moreover, depending on the condensation environment (e.g., $Si(OH)_4$ concentration, temperature, pH), the silica condensate can differ significantly in density and composition (Perry et al., 2003). Si also shows strong interactions with plant biomolecules such as cellulose (Perry and Lu, 1992), phenol- or lignin-carbohydrate complexes (Inanaga and Okasaka, 1995; Inanaga et al., 1995), callose (Law and Exley, 2011), and proteins (Perry and Keeling-Tucker, 2003). Although little is known about these chemical interactions, Si possibly links to these complex plant biomolecules through dihydrogen bonds (Zhang et al., 2013). As different condensation locations are characterized by different plant biomolecules, this strong interaction with the organic plant environment may result in an additional chemical and spectral difference in silica between plant lineages, which may explain the better calibration model for graminoids separately. Plant lineages show a large variation in the absolute bSi concentration (Hodson et al., 2005), possibly

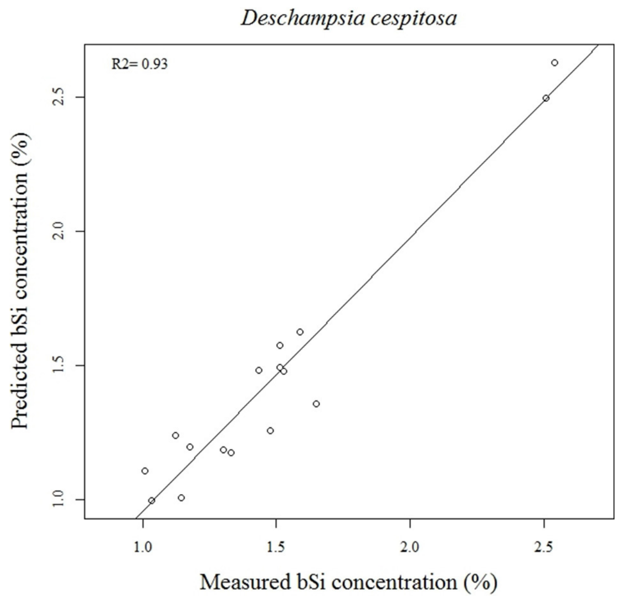

FIGURE 7 | Validation plot of the NIRS calibration model based on samples of *Deschampsia cespitosa*.

also influencing the characteristics of the individual Si condensates and its interactions with the organic environment. Finally, the three calibration models may also be confounded by other plant compounds as lignin, cellulose, and phenolics, which are, in some plant families, closely related to plant silica (Schoelynck et al., 2010; Cooke and Leishman, 2012). However, our dataset was not large enough to compare NIRS calibration model characteristics for different plant groups and further research is needed to assess the importance of plant group/species specific calibrations.

METHOD EFFICIENCY AND COST, AND FUTURE CHALLENGES

Using NIRS for plant Si analysis significantly reduces the operational and time costs. The time for sample drying is dependent on the amount of samples dried at the same time, but in all cases it is small and identical for both methods, and is therefore omitted from this estimation. In both methods, it took about 5 min to grind each sample using a ball-mill. In the reference method, about 30 mg of each plant sample was weighed, bSi was extracted in an alkaline solution and the extract was analyzed spectrophotometrically. Because this reference method was already highly optimized, e.g., by using an automated spectrophotometric device (SKALAR, 332 samples per run), each of these three steps only took about 3 min. When plant Si was analyzed by using NIRS scanning, it took 3 min per sample for the production of a tablet and 1.5 min per sample for the NIRS scan itself. As such, our calibration shows that the use of NIRS may reduce the analysis time by at least 30% compared to the chosen reference method for plant Si analysis, which is comparable to that reported for XRF spectroscopy (Reidinger et al., 2012). However, most time was still spent on the pre-treatment (drying, grinding, making tablet) of the plant sample. We believe that this pre-treatment may explain our strong calibration across largely differing plant species. However, omitting the grinding and rather extending the NIRS method to dried unground plant samples may be worthwhile as it results in a considerable progress in time efficiency, reducing the analysis time to 1.5 min after drying (90% reduction). The application to unground samples would introduce the structure of the plant leaf as an important calibration factor. Leaf structure strongly differs between plant species. Although a scatter correction of the NIR spectra may reduce the influence of structure (e.g., Gislum et al., 2004), the earlier described plant family specific location of silica deposition probably requires separate calibrations for each plant family or even plant species. Application of NIRS on fresh plant material, which would exclude all sample pre-treatment and allow for *in situ* non-destructive field monitoring of plant silicon, shows some larger challenges as all wavelength zones with high influence on our calibration model were situated in zones typical for O–H bonds of water, which should be avoided when working with fresh plant samples (e.g., Foley et al., 1998; Morón et al., 2007). Finally, we recommend other studies to carefully consider whether washing of the plant samples is needed in order to remove dust/soil particles. This will extend the analysis time, but is a common factor for both the chemical and the spectral analysis method.

An even stronger gain in efficiency and reduction in operational costs is obtained when applying NIRS for simultaneous analysis of different plant compounds. As a result of its central role in the functioning of plants, Si biochemistry in plants is closely linked to that of numerous other chemical compounds (Cooke and Leishman, 2011). Studies that quantify Si in plants often also quantify plant structural compounds such as lignin and cellulose (Schoelynck et al., 2010), as well as compounds related to abiotic and biotic plant stress (toxic metals, secondary metabolites) and plant macronutrients (e.g., N and P). Numerous studies have shown the applicability of NIRS for the analysis of many of these Si-related plant compounds (e.g., Joffre et al., 1992; Gillon et al., 1999; Stolter et al., 2006; González-Martín et al., 2007). Especially in studies where focus is on different plant compounds, the advantages of using NIRS as a method are unprecedented both in efficiency and cost.

CONCLUSIONS

Our study shows that NIRS can be used to analyze silicon in plants, and as such represents a new promising method for measuring Si more effectively, at least 30% faster, and at minimal operational cost. We showed the applicability of a calibration model based on plant species across different plant families, and that accuracy can be gained by applying plant group or species-specific calibration models. Although XRF spectroscopy offers comparable advantages, we regard XRF and NIRS to be complementary with regards to the possibility of simultaneously analyzing elementary and organic compounds, respectively. As such, both methods provide scope for studies characterizing variation of bSi in natural ecosystems at much larger scales than previously possible with traditional chemical digestion methods and will open avenues for doing research on the role of Si in plants and ecosystems.

AUTHOR CONTRIBUTIONS

Kari Anne Bråthen developed the research hypothesis and the study design. Adriaan Smis performed field sampling and chemical analysis, Francisco Javier Ancin Murguzur performed NIR scanning, calibration model development, and statistical analysis. The final manuscript is the end product of joint writing efforts of all authors.

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Silicon reduces impact of plant nitrogen in promoting stalk borer (*Eldana saccharina*) but not sugarcane thrips (*Fulmekiola serrata*) infestations in sugarcane

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The stalk borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae) is a major limiting factor in South African sugarcane production, while yield is also reduced by sugarcane thrips *Fulmekiola serrata* Kobus (Thysanoptera: Thripidae). Borer management options include appropriate nitrogen (N) and enhanced silicon (Si) nutrition; the effect of N on sugarcane thrips is unknown. We tested the effects of these nutrients, in combination with resistant (N33) and susceptible (N27) sugarcane cultivars, on *E. saccharina* and *F. serrata* infestation. Two pot trials with three levels of N (60, 120, and 180 kg ha^{-1}) and two levels each of calcium silicate and dolomitic lime (5 and 10 t ha^{-1}) were naturally infested with thrips, then artificially water stressed and infested with borer. Higher N levels increased borer survival and stalk damage, while Si reduced these compared with controls. Silicon significantly reduced stalk damage in N27 but not in N33; hence, Si provided relatively greater protection for susceptible cultivars than for resistant ones. High N treatments were associated with greater thrips numbers, while Si treatments did not significantly influence thrips infestation. The reduction in borer survival and stalk damage by Si application at all N rates indicates that under field conditions, the opportunity exists for optimizing sugarcane yields through maintaining adequate N nutrition, while reducing populations of *E. saccharina* using integrated pest management (IPM) tactics that include improved Si nutrition of the crop and reduced plant water stress. Improved management of N nutrition may also provide an option for thrips IPM. The contrasting effects of Si on stalk borer and thrips indicate that Si-mediated resistance to insect herbivores in sugarcane has mechanical and biochemical components that are well developed in the stalk tissues targeted by *E. saccharina* but poorly developed in the young leaf spindles where *F. serrata* occurs.

Keywords: plant nutrition, nitrogen fertilizer, sugarcane cultivars, calcium silicate, integrated pest management, silicon-mediated resistance, thrips, stalk borer

INTRODUCTION

Lepidopteran stalk borers are major pests of sugarcane in almost all regions of the world where this crop is grown (Leslie, 2004). In South Africa, the indigenous pyralid borer *Eldana saccharina* Walker has been the crop's major pest since the early 1970s after it invaded sugarcane from its natural wetland host plants and gradually spread through the sugar industry (Carnegie, 1974; Atkinson et al., 1981). The direct loss in revenue for cane farmers due to borer activity reducing stalk sucrose content is estimated at US$25,760,000 annum^{-1}; however, the indirect costs associated with harvesting the crop when it is only 12 months old (a strategy designed to minimize stalk borer damage), rather than at 15–18 months during its maximum sucrose accumulation period, are more substantial and estimated at US$63,290,000 annum^{-1} (Rutherford, unpublished data). This makes the total annual loss to the industry about US$89,050,000 (Baker, 2014). Nonetheless, early harvesting to avoid the build-up of economically damaging infestations, along with other cultural practices, most important of which is the use of resistant cultivars (Keeping, 2006; Ramburan et al., 2009), remain the most widely used tactics for managing the borer. The insecticide, α-cypermethrin, has also been deployed with noteworthy success to suppress borer populations in cane that is to be aged or "carried over" from one milling season to the next (Leslie, 2009).

Another commonly used cultural control measure has been to reduce applications of nitrogen (N) fertilizer. The effects of N on host plant nutritional quality and herbivorous insect survival and growth have been widely studied (e.g., Mattson, 1980; White, 1984; Coley et al., 2006). In sugarcane, infestations of *E. saccharina* can be exacerbated by high plant N and water stress (Atkinson and Nuss, 1989). Growers were therefore encouraged to reduce their fertilizer applications by 10–30 kg N ha^{-1}, depending on the N mineralization potential of the soil, and the likelihood of water stress and borer infestation (Anon, 2005). There is, however, a yield penalty associated with this practice and there is growing recognition that farmers need to revert to recommended rates of N fertilizer that will optimize yields but not incur the risk of heavy borer damage (Stranack and Miles, 2011; Rhodes et al., 2013).

With these factors in mind, integrated pest management (IPM) of *E. saccharina* needs to incorporate plant nutrition practices that render the crop less attractive to or less supportive of the borer, while simultaneously providing beneficial (or at least no detrimental) effects on crop growth and yield (Keeping et al., 2013). Thus, a balance needs to be sought between these two potentially opposing requirements. One way to address this balance is through the enhancement of the crop's silicon (Si) status. Silicon has historically been neglected in plant nutrition due to its non-essentiality in higher plants (Epstein, 1999), but its fundamental importance in amelioration of abiotic and biotic stresses, is now beyond doubt (Epstein, 2009). Of the biotic stresses that Si can alleviate, insect herbivores and fungal pathogens are especially prominent (Reynolds et al., 2009; Romero et al., 2011); and in Si-accumulating crops such as sugarcane, the capacity of Si to constrain damage by stalk borers, including *E. saccharina*, has now been well documented (Reynolds et al., 2009). There are several mechanisms whereby Si can mediate plant defense against insect herbivores: (1) increased physical (passive) resistance due to amorphous silica deposited in plant tissues, leading to their reduced digestibility and/or increased hardness and abrasiveness (Massey et al., 2006; Massey and Hartley, 2009); (2) active priming of plant chemical defenses by soluble Si and its interaction with the jasmonate (JA) signaling pathway, facilitating enhanced production of defensive enzymes (Gomes et al., 2005; Costa et al., 2011; Ye et al., 2013); (3) indirect defense based on augmented release of herbivore-induced plant volatiles (HIPVs) that attract natural enemies of the attacking herbivore (Kvedaras et al., 2010). In sugarcane, Si-mediated resistance to *E. saccharina* includes physical resistance to stalk penetration by young larvae associated with silica deposits in the stalk epidermis, leading to increased mortality and slower larval growth (Kvedaras and Keeping, 2007; Keeping et al., 2009). However, priming of plant chemical defenses, as in (2) above cannot be excluded.

Most of the earlier work on Si nutrition in sugarcane focused on yield responses (which in many instances were substantial) to application of Si-rich materials to low-Si soils (e.g., Ayres, 1966; Anderson et al., 1991; Berthelsen et al., 2001a). Hence, in South Africa, provision of Si to this crop in regions where its endogenous availability is limited could potentially deliver yield improvements that derive from both its direct effects on plant growth and its indirect effects in suppressing borer damage. The improved (Si-mediated) resistance of the crop to borer may also facilitate a return to recommended rates of N for optimal crop growth. A recent field study by Rhodes et al. (2013) showed that *E. saccharina* responded positively to increasing rates of N in only a minority of cases, supporting the argument that reducing N rates to levels below those required for optimum growth is not warranted.

Support for the idea that Si could be used to offset the promotional effects of N on *E. saccharina* development in sugarcane was first presented by Meyer and Keeping (2005), based on a preliminary potted cane study including five cultivars. Maximum reductions in percent stalk length bored using Si amendment at 200 kg ha^{-1} ranged from 70% at the lowest N level (60 kg ha^{-1}), to 39% at the intermediate N level (120 kg ha^{-1}) and 35% at the highest N treatment (180 kg ha^{-1}). However, their experimental design did not allow for analyses of independent or interactive effects of N and Si. Sétamou et al. (1993) tested the effects of N and Si in separate experiments on the bionomics of *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) in maize; their findings were consistent with the hypothesis that Si may reduce insect performance (by increasing plant resistance) under conditions of high N fertilization. Hanisch (1981) reported that increasing rates of N and Si augmented and suppressed, respectively, reproduction in *Sitobion avenae* (F.) and *Metopolophium dirhodum* (Walker, Homoptera: Aphididae) on wheat plants, but did so differentially depending on aphid species. Nabity et al. (2012) similarly found that N and Si fertilization of three energy crops differentially affected leaf consumption by two insect herbivores and that consumption depended on herbivore tolerance of high Si diets. Such findings emphasize the likely different outcomes that a plant-nutritional IPM approach could have on different pests (especially from different feeding guilds) attacking the same crop (Keeping and Kvedaras, 2008), and that crop nutrition could possibly be tailored according to pest prevalence in different areas with different soils and climate to achieve optimum yields.

The management of pests in South African sugarcane was further complicated by the appearance of the exotic sugarcane thrips *Fulmekiola serrata* Kobus (Thysanoptera: Thripidae) in 2004 (Way et al., 2006b) and its subsequent establishment throughout the industry. The insect attacks the young leaves emerging at the top of the plant, where its sap-sucking activity causes leaf yellowing, desiccation and binding together of the leaf tips (Williams, 1956; Way et al., 2006a). Measured yield reductions (tons sucrose ha^{-1}) of between 16 and 24% (Way et al., 2010) can probably be attributed partly to loss of photosynthetic activity in the damaged leaves. Information on this pest is scant, even from its oriental region of origin, and no conclusive studies have been conducted on its response to plant nutrition. Preliminary work by Keeping et al. (2010), however, indicated that Si provision to potted sugarcane had no effect on the number of thrips recovered from plants. Current control measures include cultivar resistance, manipulation of planting dates to avoid summer thrips population peaks, and systemic insecticides (Keeping et al., 2008; Leslie and Moodley, 2011).

The primary aim of the present study was to investigate the independent (i.e., main) and interactive effects of plant nitrogen and silicon on *E. saccharina* and *F. serrata*, and to establish whether these effects interacted in any way with sugarcane cultivar as a treatment. Previous work has shown that Si has a greater effect in protecting susceptible sugarcane cultivars against borer attack, especially when plants are water stressed (Kvedaras et al., 2007b). The possibility therefore exists that N has a differential effect on resistance of genotypically susceptible and resistant cultivars to one or both pests, as does the possibility that Si differentially affects plant resistance depending on plant N status and the insect herbivore involved. For *F. serrata*, nothing is known of its response to N in any plant. As Si frequently offers little or no benefit to unstressed plants (Epstein, 2009), and because water stress increases susceptibility of sugarcane to *E. saccharina* (Atkinson and Nuss, 1989) but amplifies the effect of Si in protecting against the borer (Kvedaras and Keeping, 2007), we induced water stress *equally* in all plants before borer infestation (see below) to allow clear differentiation of treatment effects.

The results presented here were initially published in condensed form as an un-refereed short communication (Keeping et al., 2012) in the *Proceedings of the Annual Congress of the South Africa Sugar Technologists' Association* (ISSN 1028-3781).

MATERIALS AND METHODS

As our study was aimed at testing the principles detailed above, we chose to conduct experiments using potted sugarcane, where extraneous conditions and infestation levels of *E. saccharina* could be better controlled than in field trials. Importantly, we also wished to impose artificial water stress over the period of *E. saccharina* infestation, which necessitated exclusion of rainfall from the plants. The trials were therefore established in a shade house (14 m × 25 m × 3.3 m) with clear polycarbonate roofing and walls of 40% green-shade cloth, at the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, South Africa (29°42′0″ S; 31°2′0″ E), over two successive seasons (early December 2009 and 2010). Planting the trials in summer ensured exposure of the young plants to natural infestation by sugarcane thrips, which preferentially attack young plants (Keeping et al., 2008) and reach a peak in population numbers during December and January in KwaZulu-Natal, South Africa, whereafter numbers drop rapidly going into winter (Way et al., 2007).

TREATMENTS AND DESIGN

Sugarcane transplants were produced from single-budded setts, cut from mature stalks of two commercial cultivars, one relatively resistant (N33) and the other susceptible (N27) to both *E. saccharina* and *F. serrata*. One-month-old transplants of each cultivar were planted into 25 L PVC pots (three seedlings per pot), with perforated bases, containing 31 kg (dry weight) of clean, sieved and thoroughly leached river sand. Silicon treatments were applied before planting by thoroughly mixing a calcium silicate slag (Calmasil®, supplied by PDB Holdings, Pty (Ltd), Middelburg, RSA) into the sand at rates equivalent to 5 t ha^{-1} and 10 t ha^{-1}. Calmasil has a Si content of 10.3%, with a neutralizing capacity of 101% of that of calcium carbonate; it also contains 29.5% calcium (Ca) and 6.7% magnesium (Mg). In order to balance for the effect of Calmasil on sand pH, and Ca and Mg supply, controls (with no Si treatment) received equivalent amounts of 5 t ha^{-1} and 10 t ha^{-1} of thoroughly incorporated dolomitic lime, containing 21.0% Ca and 8.1% Mg.

Four pots, containing each of the above four soil treatments, were placed linearly into a total of 48 galvanized metal troughs (200 cm long × 40 cm wide × 10 cm deep). Nutrient solution placed in the troughs supplied the N treatments in the form of ammonium sulfate and di-ammonium phosphate at three different N rates equivalent to 60 kg ha^{-1} ("N1"), 120 kg ha^{-1} ("N2"), and 180 kg ha^{-1} ("N3"). The solution also supplied adequate amounts of potassium, phosphorus, and sulfur (as potassium sulfate, potassium phosphate, and potassium chloride), as well as additional Ca and Mg. Two liters of a 20 L stock solution containing these chemicals were added to 20 L water in each trough and the latter then topped up with 18 L water to a total of 40 L per trough. Every fortnight, the troughs were emptied and fresh nutrient solution supplied as above. Micronutrients (Hygrotech® Micronutrient Hydroponic Seedling Mix) were supplied in solution at a rate of 1.0 g L^{-1} water per pot, added directly to each pot by hand the day after the N solutions were changed. "Skirts" of black plastic sheeting (250 μm thick) were placed over the troughs, with holes cut to fit tightly around the pots, to reduce evaporation and prevent algal growth. Although restriction of root growth in pot experiments is unavoidable, the unlimited supply of water and nutrients during the growth phase (i.e., before water stressing) and the ability of roots to egress into the water troughs through holes in the pots would have assisted in reducing pot binding.

For Trial 1 (2009), the N treatments were introduced 10 weeks after the trial was planted. Until that stage, pure water was supplied via the troughs, while an N:P:K fertilizer was applied to the sand surface once at planting (16 g L^{-1} water per pot) and nutrient seedling mix was supplied fortnightly in solution at 160 g L^{-1} water per pot. For Trial 2 (2010), the N treatments commenced at planting and the seedling mix applied fortnightly as above when the N solutions were renewed.

Pots were arranged in a split-split-plot design with eight replications, where whole plot was "cultivar" (=three troughs in a row), sub-plot was "nitrogen" (=one trough) and sub–sub plot was "silicon" (=one pot). Each row in a trial consisted of three troughs and each trial contained a total of 192 pots. Save for the differences between trials in commencement of N treatments, the treatments and replications were identical; however, the design was re-randomized.

THRIPS SAMPLING

The trials were naturally infested by *F. serrata* entering the shade houses from the surrounding field-grown sugarcane. At 3 month's age, all pots in both trials were sampled for *F. serrata* by removing the leaf spindle (i.e., the young fully furled leaves at the apex of the plant) plus adjacent first unfurled leaf from one plant per pot. Spindles plus first leaf were collected into plastic Ziploc bags, which were immediately transferred to a freezer (–24°C) before assessment at a later time. Thrips numbers were assessed by washing the thrips off the spindle with warm water and a few drops of detergent in a plastic tray, and counting their numbers (nymphs plus adults per spindle) under a dissecting microscope. Trial 1 was assessed only for the effects of Si (not N) on thrips numbers in the leaf spindle.

Following the thrips sampling, the trials were sprayed with insecticide monthly (chlorpyriphos 2 mL L^{-1} water) to prevent feral infestations of *E. saccharina* and other pests (aphids, scale, leafhoppers). Spraying was halted 2 months before inoculation with *E. saccharina* (see below) to allow time for pesticide residue on the plants to degrade.

LEAF AND SOIL SAMPLING

At four month's age, third leaf (top visible dewlap or TVD) samples for nutrient analysis were taken from each plant in every pot, and leaves from pots with identical treatment combinations were combined between two adjacent replicates. This produced four samples per treatment combination to obtain sufficient leaf material for analysis. Leaf blades were stripped from the midrib and the blades dried, ground, and submitted for N, P, K, Ca, and Si analysis. Trial 1 was also sampled at 10 months.

Soil samples were taken from the trials at 5.5 months to establish responses in levels of soil Ca, Mg, Si, and pH (in 0.01 M calcium chloride) to the Calmasil and lime treatments. Silicon was extracted using 0.02 N sulphuric acid (Kanamugire et al., 2006) for Trial 1 and 0.01 M $CaCl_2$ (Berthelsen et al., 2001b; Miles et al., 2011) for Trial 2; Si concentrations were determined using the ammonium molybdate method (Fox et al., 1967). The change in Si extraction method followed routine changes to soil analysis, including improved soil Si determination with $CaCl_2$ extraction (Miles et al., 2011).

WATER STRESSING

At 7 months, the troughs were removed from beneath the pots and the latter placed directly onto the gravel floor of the shade house in their exact original location. Thereafter, the pots were drip irrigated for 15 min daily for one week using 2 L h^{-1} pressure-compensating drippers (=1.0 L pot^{-1} day^{-1}). Irrigation was then reduced weekly to 10 min (666 mL pot^{-1}), 7 min (466 mL pot^{-1}), 5 min (333 mL pot^{-1}) and 3 min (200 mL pot^{-1}) per day to impose an incremental increase in water stress *across all pots* over 4 weeks (i.e., it did not constitute a treatment). Water stress promotes *E. saccharina* larval survival and development (Atkinson and Nuss, 1989) and ensures a level of infestation sufficient to clearly discriminate treatment effects (Keeping, 2006; Kvedaras et al., 2007b). Care was taken to ensure that a minimum of four green leaves remained on all plants (Inman-Bamber, 2004), by supplying additional water if necessary.

BORER INFESTATION AND HARVEST

After 4 weeks of increasing water stress (about 8 month's age), the trials were artificially infested with *E. saccharina*. This entailed careful inoculation of plants with batches of 150 fertilized eggs (laid on small pieces of paper towel) placed behind a lower leaf sheath of one primary tiller in each pot (Keeping, 2006). At the time of inoculation, most of the eggs were in the "black head" stage of development and hatched within 24 h, reducing exposure to ant predation. Surviving larvae boring into the stalks were allowed to develop for 9 weeks in Trial 1 and for 12 weeks in Trial 2. This equated to about 600 degree-days of development, measured using Tempest® Degree-day Units placed in the trials (Insect Investigations Ltd, Cardiff, UK; $t = 10°C$), by which time the majority of larvae were present as late instars (Way, 1995) and the trials could be harvested.

At harvest, stalks from every pot were removed at the base and their length determined. Stalks were then bisected lengthwise to extract and count all surviving larvae and pupae, and to establish the length of stalk bored. The percentage of total stalk length bored was used as a measure of borer damage in the analysis.

DATA ANALYSIS

All data were tested for univariate normality (Anderson Darling or Shapiro–Wilk tests) and homogeneity of variance (Bartlett's test) prior to analysis of variance (ANOVA). Where conditions for parametric analysis were not met, appropriate transformations (log or square root) were applied. Where data were unbalanced, as in the case of the leaf Si data for Trial 1 (at 4 months of age), a Residual Maximum Likelihood Ratio (REML) or General Linear Mixed Model (GLMM, with a negative binomial distribution) analysis was performed instead of ANOVA, and only main effects were tested. Probability (p) values in the text are derived from these analyses, unless stated otherwise. Where these initial analyses yielded significant differences between treatments, Holm–Sidak *post hoc* tests were applied to determine location of differences.

Effects of cultivar and nitrogen treatments on soil Ca, Mg, Si, and pH are excluded from the results, as the latter were intended only to provide a base-line indication of soil properties of importance to our aims. Similarly, effects of cultivar and N on leaf Ca, Mg, and Si are excluded as these were beyond the scope of the study; analyses revealed that no significant interactions occurred between the main effects.

RESULTS

SOIL ANALYSES – TRIAL 1

Calmasil resulted in significantly higher levels of soil Ca, Mg, and Si than lime, although differences in Ca and Mg between the 5 and 10 t ha^{-1} rates of each treatment were non-significant (**Table 1**). Soil Si from the 10 t ha^{-1} Calmasil treatment was 84% higher than that from Calmasil at 5 t ha^{-1} (**Table 1**). There were no significant differences in soil pH between lime and Calmasil treatments, although means for Calmasil were somewhat higher than for lime (**Table 1**).

SOIL ANALYSES – TRIAL 2

Calcium and Mg levels in Trial 2 were similar to Trial 1; however, Si values were substantially lower for the Calmasil treatments in Trial 2 due to the $CaCl_2$ extraction method, which gives a more reliable estimate of plant-available Si. There were also no significant differences in soil Mg (**Table 1**). In this instance, soil pH was significantly higher for both rates of Calmasil than for lime, but differences between rates within each treatment were not significant (**Table 1**).

LEAF ANALYSES – TRIAL 1

Cultivars did not differ in leaf Si content ($p = 0.7$; 2.4 ± 0.2 g kg^{-1} for N27 and N33). However, leaf N was significantly higher in N27 (17.4 ± 0.5 g kg^{-1}) than in N33 (15.1 ± 0.3 g kg^{-1}; $p < 0.001$). Leaf N increased significantly ($p < 0.001$) between all N treatment rates from 14.2 ± 0.3 g kg^{-1} (N1), through 16.1 ± 0.5 g kg^{-1} (N2), to 18.4 ± 0.5 g kg^{-1} (N3; Holm–Sidak, $p < 0.05$).

Leaf Ca and Si were both significantly higher in the Calmasil than in the lime treatments; leaf Si in the 10 t ha^{-1} Calmasil treatment was also significantly (42%) higher than Calmasil at 5 t ha^{-1} (**Table 2**). There was no significant effect of the Calmasil and lime treatments on leaf Mg content (**Table 2**).

LEAF ANALYSES – TRIAL 2

There was no effect of cultivar on leaf Si ($p = 0.95$; 3.4 ± 0.3 g kg^{-1} for N27 and N33) or on leaf N ($p = 0.34$; N27: 15.7 ± 0.2 g kg^{-1},

Table 1 | Soil analysis for Trials 1 and 2 at 5.5 months in response to lime and Calmasil treatments.

Treatment	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Si (mg kg^{-1})*	pH ($CaCl_2$)
Trial 1				
Lime 5 t ha^{-1}	397 ± 30 a	90 ± 4 a	11.0 ± 3.1 a	5.7 ± 0.5
Lime 10 t ha^{-1}	442 ± 22 a	99 ± 8 ab	12.0 ± 3.6 a	6.0 ± 0.8
Calmasil 5 t ha^{-1}	822 ± 21 b	158 ± 5 bc	50.0 ± 4.6 b	6.8 ± 0.1
Calmasil 10 t ha^{-1}	896 ± 86 b	137 ± 16 c	95.7 ± 6.9 c	7.0 ± 0.3
p value	<0.001	0.002	<0.001	0.34
Trial 2				
Lime 5 t ha^{-1}	593 ± 45 a	98 ± 15	6.9 ± 0.6 a	7.1 ± 0.1 a
Lime 10 t ha^{-1}	868 ± 91 ab	127 ± 28	6.9 ± 0.5 a	7.2 ± 0.1 a
Calmasil 5 t ha^{-1}	1019 ± 80 bc	106 ± 20	15.2 ± 0.6 b	7.8 ± 0.1 b
Calmasil 10 t ha^{-1}	1277 ± 143 c	99 ± 8	19.9 ± 0.7 c	8.0 ± 0.1 b
p value	<0.001	0.66	<0.001	<0.001

*Values are means ± standard error (decimal figures are given only where deemed necessary). Probability (p) values are from ANOVA. Means within the same column followed by the same letter are not significantly different (Holm–Sidak, $p < 0.05$). *Silicon extracted using H_2SO_4 in Trial 1 and $CaCl_2$ in Trial 2.*

Table 2 | Leaf analysis for Trials 1 and 2 at 4 months (Ca, Mg, Si) and 10 months (Si only) in response to lime and Calmasil treatments.

Treatment/statistic	Ca (g kg^{-1})	Mg (g kg^{-1})	Si (g kg^{-1}) 4 months	Si (g kg^{-1}) 10 months
Trial 1				
Lime 5 t ha^{-1}	1.8 ± 0.2 a	1.0 ± 0.03	1.7 ± 0.2 a	–
Lime 10 t ha^{-1}	1.9 ± 0.2 a	0.9 ± 0.04	1.6 ± 0.2 a	–
Calmasil 5 t ha^{-1}	2.5 ± 0.3 b	1.0 ± 0.03	2.6 ± 0.3 b	–
Calmasil 10 t ha^{-1}	2.7 ± 0.3 b	1.0 ± 0.03	3.7 ± 0.3 c	–
p value	0.009	0.24	<0.001	–
Trial 2				
Lime 5 t ha^{-1}	2.8 ± 0.1	1.2 ± 0.03	2.7 ± 0.2 a	1.9 ± 0.1 a
Lime 10 t ha^{-1}	2.9 ± 0.1	1.2 ± 0.04	2.6 ± 0.1 a	1.8 ± 0.5 a
Calmasil 5 t ha^{-1}	3.1 ± 0.1	1.3 ± 0.05	3.8 ± 0.1 b	4.1 ± 0.2 ab
Calmasil 10 t ha^{-1}	2.9 ± 0.1	1.2 ± 0.04	4.3 ± 0.2 b	5.8 ± 1.1 b
p value	0.06	0.07	<0.001	0.004

Values are means ± standard error. Probability (p) values are from ANOVA. Means within the same column followed by the same letter are not significantly different (Holm–Sidak, $p < 0.05$).

N33: 15.2 ± 0.2 g kg^{-1}). Leaf N did not respond to the N treatments in either the 4-month ($p = 0.97$) or 10-month ($p = 0.08$) samples. However, at 10 months mean leaf N concentration increased from 9.7 ± 0.8 g kg^{-1} (N1), through 10.4 ± 0.3 g kg^{-1} (N2), to 12.1 ± 0.5 g kg^{-1} (N3).

Leaf Ca and Mg content were not affected by Calmasil and lime treatments (**Table 2**). At 4 months, Calmasil produced significantly higher leaf Si than lime, but the 5 and 10 t ha^{-1} rates did not differ significantly within treatments (**Table 2**). Similar results were recorded at 10 months. Notably, leaf Si content increased from 4 to 10 months in the Calmasil treatments but decreased in the lime treatments (**Table 2**).

THRIPS INFESTATION

In both trials, cultivar N33 had significantly fewer thrips (*F. serrata*) than N27 (**Table 3**); numbers on N33 were 19% lower in Trial 1 and 32% lower in Trial 2. In Trial 2, the N2 and N3 treatments significantly increased thrips number per spindle over N1 (**Table 3**). The soil treatments (and therefore Si) had no significant effects on thrips abundance (**Table 3**), and there were no significant interactions.

BORER INFESTATION – TRIAL 1

Cultivar significantly affected number of borers (*E. saccharina*) recovered ($p < 0.001$), being 83% lower in N33 (0.2 ± 0.03 borers $stalk^{-1}$) than in N27 (1.2 ± 0.09 borers $stalk^{-1}$). There was a

Table 3 | Thrips abundance in Trials 1 and 2 at 3 months in response to cultivar, soil, and nitrogen treatments.

Treatment/statistic	Total thrips spindle^{-1}	
	Trial 1	**Trial 2**
Cultivar		
N27	32.2 ± 2.6	22.3 ± 1.5
N33	26.2 ± 1.9	15.2 ± 1.4
p value	0.03	0.01
Nitrogen		
60 kg ha^{-1} (N1)	–	14.13 ± 1.6 a
120 kg ha^{-1} (N2)	–	20.36 ± 1.6 b
180 kg ha^{-1} (N3)	–	21.77 ± 2.2 b
p value	–	0.002
Soil		
Lime 5 t ha^{-1}	31.2 ± 3.7	21.94 ± 2.7
Lime 10 t ha^{-1}	29.3 ± 3.2	18.56 ± 1.9
Calmasil 5 t ha^{-1}	30.2 ± 3.3	16.25 ± 1.6
Calmasil 10 t ha^{-1}	26.3 ± 2.6	18.25 ± 2.2
p value	0.51	0.23

Values are means ± standard error. Values for N treatments in Trial 1 are not provided for reasons given in text. Probability (p) values are from ANOVA. Means within the same column followed by the same letter are not significantly different (Holm–Sidak, $p < 0.05$).

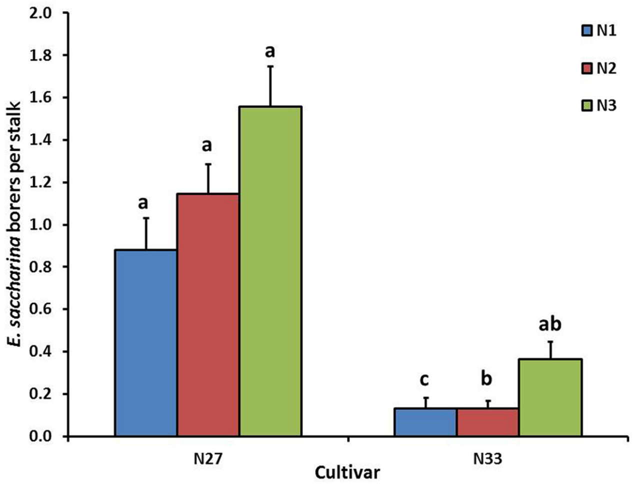

FIGURE 1 | Mean (±SE) number of *E. saccharina* recovered per stalk from sugarcane cultivars (N27 and N33) fertilized at different N rates (N1, N2, and N3) in Trial 1. Bars with the same letter/s above them do not differ significantly (Holm–Sidak test, $p < 0.05$).

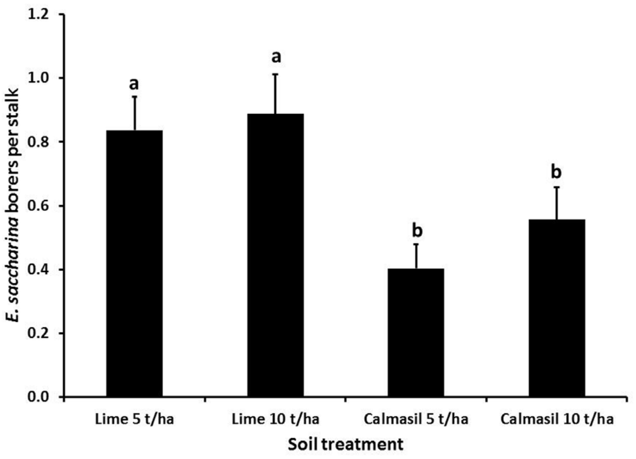

FIGURE 2 | Mean (±SE) number of *E. saccharina* recovered per stalk from sugarcane amended with Calmasil and lime at two different rates in Trial 1. Bars with the same letter/s above them do not differ significantly (Holm–Sidak test, $p < 0.05$).

significant interaction between cultivar and nitrogen ($p = 0.03$); although N did not significantly increase borer numbers in N27, there was a significant increase in N33 between N1 and N3, to the extent that the N33 + N3 treatment did not differ significantly from N27 (**Figure 1**). Soil treatments significantly affected borer numbers ($p = 0.018$), which were reduced by an overall 44% in the Calmasil treatments compared with lime (**Figure 2**).

Percent stalk length damaged was significantly reduced ($p < 0.001$) by 88% in N33 (1.0 ± 0.1%) compared with N27 (8.7 ± 1.2%). Nitrogen treatments significantly affected percent stalk length bored ($p = 0.008$), the latter increasing from 2.8 ± 0.5% in N1, through 4.6 ± 1.2% in N2, to 6.1 ± 1.0% in N3; the difference between N1 and N3 was significant (Holm–Sidak, $p < 0.05$). There was a significant interaction between cultivar and soil treatment ($p = 0.003$), wherein the Calmasil treatments had no effect on borer damage in N33, but significantly reduced damage in N27; notably, damage in N27 with Calmasil 5 t ha^{-1} did not differ significantly from that in N33 with lime 5 t ha^{-1} (**Figure 3**).

BORER INFESTATION – TRIAL 2

Borer numbers were significantly reduced (by 63%; $p < 0.001$) from 4.6 ± 0.6 borers stalk^{-1} in N27 to 1.7 ± 0.3 borers stalk^{-1} in N33. Nitrogen treatment significantly affected borer recovery ($p < 0.001$), with numbers per stalk increasing from 1.6 ± 0.4 in N1, through 2.9 ± 0.5 in N2, to 4.8 ± 0.8 in N3. Numbers in both N2 and N3 differed significantly from N1 (Holm–Sidak, $p < 0.05$), but not from one another. Soil treatments had a significant effect on borer numbers ($p < 0.001$), wherein the 10 t ha^{-1} Calmasil treatment reduced numbers significantly compared with all other treatments and by 41% compared with 10 t ha^{-1} lime; Calmasil at 5 t ha^{-1} did not reduce numbers compared with lime controls (**Figure 4**). There were no significant interactions between treatments.

Percent stalk length damaged was significantly reduced (by 54%; $p < 0.001$) from 7.4 ± 0.8% in N27 to 3.4 ± 0.4% in N33. Nitrogen treatments significantly affected percent stalk length bored ($p < 0.001$), the latter increasing from 3.5 ± 0.5% in N1, through 5.5 ± 0.7% in N2, to 7.2 ± 0.9% in N3; both N2 and N3 differed significantly from N1, but not from one another

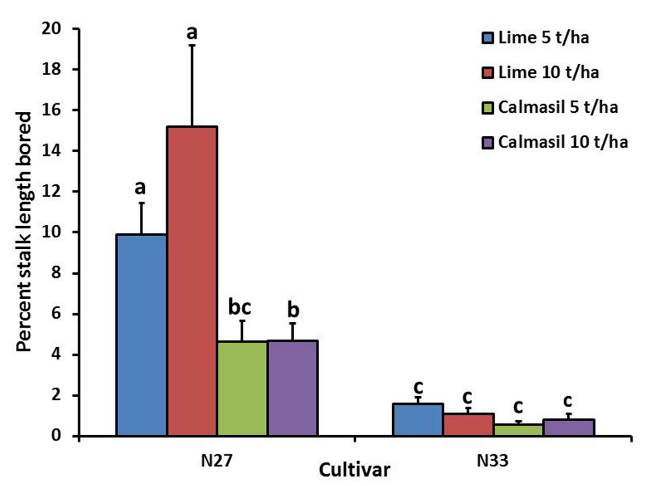

FIGURE 3 | Mean (±SE) percent stalk length bored by *E. saccharina* in sugarcane cultivars (N27 and N33) amended with Calmasil and lime at two different rates in Trial 1. Bars with the same letter/s above them do not differ significantly (Holm–Sidak test, $p < 0.05$).

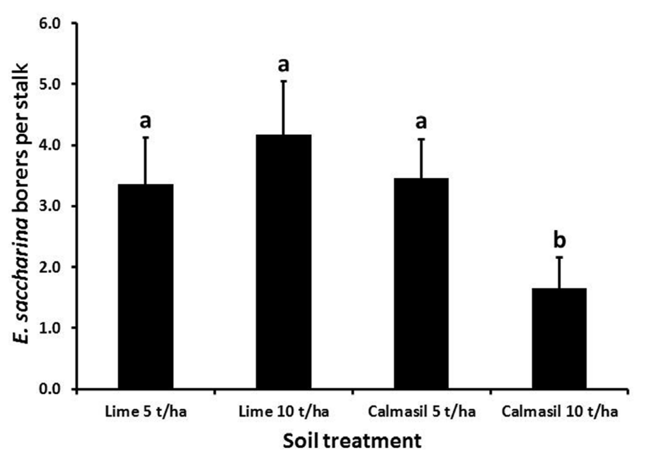

FIGURE 4 | Mean (±SE) number of *E. saccharina* recovered per stalk from sugarcane amended with Calmasil and lime at two different rates in Trial 2. Bars with the same letter/s above them do not differ significantly (Holm–Sidak test, $p < 0.05$).

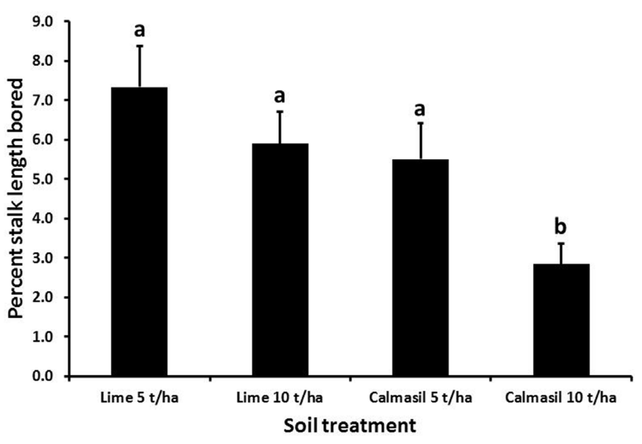

FIGURE 5 | Mean (±SE) percent stalk length bored by *E. saccharina* in sugarcane amended with Calmasil and lime at two different rates in Trial 2. Bars with the same letter/s above them do not differ significantly (Holm–Sidak test, $p < 0.05$).

(Holm–Sidak, $p < 0.05$). Borer damage was significantly affected by the soil treatments ($p < 0.001$), with percent stalk length bored reduced significantly in the 10 t ha^{-1} Calmasil treatment compared with all other treatments and by 49% compared with 5 t ha^{-1} lime; Calmasil at 5 t ha^{-1} did not reduce numbers compared with lime controls (**Figure 5**). There were no significant interactions between treatments.

DISCUSSION

Soil and leaf analyses from both trials conducted in this study demonstrated that Calmasil significantly and consistently raised soil and leaf Si content. There was also a clear positive effect of rate of application on soil and plant Si (**Tables 1** and **2**). While soil Ca and Mg levels were increased more by Calmasil than by dolomitic lime applied at the same rates (**Table 1**), the effects on leaf content of these elements were evident only for Ca in Trial 1 (**Table 2**). The most consistent and largest increases in leaf concentrations of elements provided by the Calmasil treatments were those of Si (**Table 2**). Furthermore, the leaf concentrations of Ca and Mg were within the "satisfactory" range of 1.5–3.9 g kg^{-1} and 0.8–1.9 g kg^{-1}, respectively, for South African sugarcane (Miles and Rhodes, 2013). It is unlikely, therefore, that Ca or Mg contributed to the significant effects of the Calmasil treatment on plant resistance to borer reported in this study and discussed below.

Of interest, the cultivars used in this study did not differ in Si content. Previous studies that included the resistant cultivar N33 found that its endogenous Si content was high compared with borer-susceptible cultivars such as N11 and N26 (Kvedaras et al., 2007a; Keeping et al., 2009). Cultivar differences in Si content are also well documented from other countries (e.g., Deren et al., 1993). Since cultivar differences in leaf N content were evident only in Trial 1, it is unlikely that borer responses to cultivar or to N treatments were linked to endogenous differences in cultivar N content.

Although Calmasil significantly increased soil pH above that produced by lime in Trial 2, this did not occur in Trial 1 (**Table 1**), making it unlikely that pH had an effect on leaf Si content and borer resistance. Furthermore, studies conducted in the SA sugar industry indicate that soil pH values in the range recorded here for the lime and Calmasil treatments (i.e., ≥5.5) are generally associated with satisfactory leaf Si concentrations above 10 g kg^{-1} (Van Der Laan and Miles, 2010). The pH values for the Calmasil treatments support the argument that calcium silicate may provide liming capabilities similar or superior to those of dolomitic lime, while providing ample quantities of Ca and Mg (Marafon and Endres, 2013).

There was no evidence that elevated leaf Si mitigated sugarcane thrips infestation, in agreement with the preliminary results of Keeping et al. (2010). While some studies have shown an increase in resistance to various thrips species in response to root-applied silicate treatments (Subramanian and Gopalaswamy, 1988; Almeida et al., 2008), a recent study on *Scirtothrips dorsalis* Hood (Thysanoptera: Thripidae) on pepper plants found minimal effects of Si on damage and thrips numbers recovered from infested plants (Dogramaci et al., 2013). The authors contended that this was due to inadequate accumulation of Si in the epidermis and mesophyll of the young leaves targeted by the insect. In sugarcane, Si content can increase from 4 g kg^{-1} in young leaves to more than 60 g kg^{-1} in old ones (Van Dillewijn, 1952). Analysis of Si content of TVD leaves and leaf spindles collected from the same tillers of 8-month-old field-grown cane (Keeping, unpublished data), showed significantly lower quantities ($p < 0.001$) of Si in the young spindle tissue (8.4 ± 0.5 g kg^{-1}) than in the TVD (15.3 ± 1.4 g kg^{-1}). We argue here that, as for *S. dorsalis* on pepper plants, the accumulated amorphous Si in the leaf spindle of Calmasil treated cane was insufficient to provide a suppressive effect on *F. serrata* populations and therefore unlikely to be of value in crop protection against this pest. It may also partly explain why the leaf spindle is the favored microhabitat for sugarcane thrips, together with the more protected environment and softer foliage that the spindle offers.

Inert, amorphous silica (plant opal) presents insect herbivores with mechanical difficulties in chewing, penetrating, and digesting plant tissues (Massey et al., 2006; Massey and Hartley, 2009; Reynolds et al., 2009). However, soluble Si appears also to be involved as plants attacked by insects and simultaneously fed with Si may show enhanced expression of several defensive enzymes compared with Si-untreated plants (Gomes et al., 2005). Most recently, Ye et al. (2013) showed that Si amendment amplified the JA mediated defense responses of rice to *Cnaphalocrocis medinalis* (Guenée, Lepidoptera: Pyralidae), and therefore served as a priming agent in this crucial plant defense signaling pathway. Although the JA pathway is active in sugarcane (Bower et al., 2001), monthly applications of JA to the leaves of Si-amended potted sugarcane failed to suppress *F. serrata* populations (Keeping et al., 2010), indicating that the effects of both Si and JA in eliciting biochemical defenses in young sugarcane tissue are indistinguishable.

Fertilization with N at 120 and 180 kg ha^{-1} significantly increased thrips recovery from leaf spindles, compared with the N rate of 60 kg ha^{-1}. While there is abundant evidence for the promotional effects of higher N rates on populations of other thrips, especially *Frankliniella* spp. (fam. Thripidae, e.g., Brodbeck et al., 2001; Chau et al., 2005; Atakan, 2006; Baez et al., 2011), our study is the first to report on effects of N on *F. serrata.* In sugarcane, the concentrations of N, Si, potassium (K), and phosphorus (P) show a marked decrease during the first three to four months of growth; furthermore, concentrations of N and K decrease considerably in individual leaves as they age (Van Dillewijn, 1952). The initially high levels of N in young plants and in young leaves may partly explain why *F. serrata* populations are substantially higher in young plants (1–3 months old) and why they favor the leaf spindle. Avoidance of N application at higher than recommended rates for optimal cane growth is therefore an important management requirement for sugarcane thrips. This can be integrated with other practices, such as planting well before or after the mid-summer population peaks to prevent exposure of young plants to high infestation pressure (Keeping et al., 2008), in combination with more resistant cultivars (this study, Keeping et al., 2008; Joshi et al., 2014) and carefully timed insecticide applications (Leslie and Moodley, 2011).

Our study confirms that resistant cultivars contribute substantially to managing infestations of both pests: compared to N27, *F. serrata* numbers were reduced in N33 by 19 and 32%, and *E. saccharina* numbers by 63 and 83% in Trials 1 and 2, respectively; borer damage was reduced by 54 and 88%. Although no quantitative index has been developed between yield loss and *F. serrata* numbers or leaf injury, reduced thrips numbers are associated with increased sucrose yields (Way et al., 2010), while the suppression of borer damage produces an equivalent saving in sucrose based on an established (but probably conservative) 1% sucrose lost for every 1% internodes bored (King, 1989). The existence of cultivars such as N33, with cross-resistance to both pests, is fortunate for both growers and plant breeders; the latter, as it can serve as a parent in crosses aimed at improving pest resistance in the sugarcane gene pool.

Higher rates of N fertilization and resultant higher plant N significantly increased *E. saccharina* survival (i.e., recovery) and damage in both trials, in concurrence with several pot and field studies of this borer (Carnegie, 1981; Atkinson and Nuss, 1989; Coulibaly, 1990; Meyer and Keeping, 2005; Van Antwerpen et al., 2011). Plant water stress is an additional factor that increases the mobilization and availability of N to herbivores (White, 1984), including *E. saccharina* (Atkinson and Nuss, 1989), and enhances insect performance in the majority of stem borers (Galway et al., 2004; Huberty and Denno, 2004). Furthermore, the significant interaction between cultivar and N in Trial 1 (**Figure 1**) suggests that the promotional effects of N may be such that a resistant cultivar that is simultaneously water stressed and fertilized at high N rates may be rendered as suitable for borer growth as a susceptible (water stressed) cultivar fertilized at low N rates. Similarly, in the absence of different N fertilization rates, water-stressed resistant cultivars may have similar borer-susceptibility to non-stressed, susceptible cultivars (Kvedaras et al., 2007b).

Notwithstanding these observations, several field studies and surveys from commercial cane where water stress was not controlled have found no clear relationship between N rate and *E. saccharina* infestation (Carnegie, 1981; Atkinson and Nuss, 1989; Stranack and Miles, 2011; Rhodes et al., 2013). In 16 harvested crops from 10 field trials, Rhodes et al. (2013) recorded significant increases in infestation in response to N in only three crops, despite seven of the crops growing over a severe drought period during 2010. A reduction in N rate for each trial to 20 kg ha^{-1} less than the recommended rates would not have achieved significant reductions in borer damage (Rhodes et al., 2013). Cultivar and its interaction with N (this study) and water stress (Kvedaras et al., 2007b) almost certainly play an important role in producing such variable responses to borer under field conditions. Most borer-resistant cultivars are also comparatively drought tolerant (Keeping and Rutherford, 2004); as the cultivars used in 7 of the 10 trials conducted by Rhodes et al. (2013) exhibit these characteristics, this, together with soil type, may have affected the impact of higher N rates and water stress on borer infestation.

In contrast to N, Si application significantly reduced borer survival (**Figures 2** and **4**) and damage (**Figures 3** and **5**) in both trials. In Trial 1, the effect of Si on borer damage was contingent on cultivar, with damage significantly diminished in susceptible N27, but not in resistant N33 (**Figure 3**). A greater benefit from Si amendment of susceptible cultivars has previously been reported for *E. saccharina* (Keeping and Meyer, 2002, 2006; Kvedaras et al., 2007b) and *Mahanarva fimbriolata* Stål (Hemiptera: Cercopidae; Korndörfer et al., 2011) in sugarcane, and for *Chilo suppressalis* (Walker, Lepidoptera: Crambidae, Hou and Han, 2010) in rice. The phenomenon has also been widely reported in studies of Si-mediated resistance to plant pathogens, where Si applications have enhanced resistance of susceptible cultivars to levels similar to those of resistant ones (e.g., Datnoff et al., 2001; Rodrigues et al., 2001; Kanto et al., 2006; Fortunato et al., 2012; Shetty et al., 2012). While the cultivars used in this study did not differ in leaf Si content, earlier studies have shown that N33, treated or untreated with Si, displayed higher plant Si content than borer-susceptible cultivars, indicating that some of its resistance is attributable to endogenously higher total plant Si content (Kvedaras and Keeping, 2007; Kvedaras et al., 2007a; Keeping et al., 2009). However, the absence of a difference in total leaf Si content (which is highly correlated with stalk Si%; Keeping, unpublished data) between N33 and N27, yet significantly different responses to Si treatment, indicate that a Si-mediated resistance mechanism is at work over and above a passive silica-based mechanical barrier. We suggest that, as has been demonstrated for a sap-sucker, *Schizaphis graminum* (Rond., Hemiptera: Aphididae, Gomes et al., 2005), on wheat and a leaf-feeder, *C. medinalis* (Ye et al., 2013), on rice, insect-inducible chemical defenses primed by soluble Si may be involved in Si-mediated resistance to *E. saccharina* in sugarcane. Confirmation of this and the possible involvement of the JA pathway would extend the existence of this mechanism to a third, insect-feeding guild (stalk borers).

The high endogenous Si content of N33 (compared with susceptible cultivars like N11 and N26) together with other resistance mechanisms to *E. saccharina*, including high fiber content and stalk rind hardness (Keeping et al., 2009; Kvedaras et al., 2009), likely account for the insignificant effects of Si fertilization (**Figure 3**) on total resistance of N33 to the borer. Such observations highlight that there is little (or no) justification, even at high rates of N, for Si fertilization of resistant cultivars purely for enhancement of stalk borer resistance. However, there are soil health related reasons, in particular wide-spread deficiencies in plant-available Si, and amelioration of soil acidity and aluminum toxicity, that argue persuasively for the continued use of Si-rich liming materials in the rainfed regions of the South African sugar industry (Moberly and Meyer, 1975; Van Der Laan and Miles, 2010; Keeping et al., 2013). Beyond this, there are other benefits, including yield enhancement and improved tolerance of various abiotic stresses, especially drought (Alvarez and Datnoff, 2001), which could justify its use.

In N27, the reduction in borer survival and stalk damage by Si application at all N rates indicates that for more susceptible cultivars planted in rainfed regions, the opportunity exists for optimizing sugarcane yields through maintaining adequate N nutrition, while reducing populations of *E. saccharina* through improved Si nutrition of the crop. However, we emphasize that Si provision would constitute only one component of an IPM strategy that must embrace other available measures (Rutherford and Conlong, 2010), including reduced plant water stress. Previous studies have shown that Si is especially beneficial in water stressed cane (Kvedaras et al., 2007b), but also that the combination of water stress with high N rates provides conditions that support the development of damaging borer infestations (Atkinson and Nuss, 1989). Results from the present study are consistent with both these observations. Excessive applications of N under conditions where lower cane yields are expected are in any case uneconomic and environmentally harmful, and should therefore be avoided (Meyer et al., 2007). We argue here that attention by growers to addressing problems of Si and other nutrient deficiencies, including N and K (see Rhodes et al., 2013 for the latter), is a first step in avoiding plant stress and reducing borer infestation. A second step is to improve soil health and root growth – and hence nutrient and water uptake – by reducing soil acidity and aluminum toxicity through liming and/or calcium silicate provision (Moberly and Meyer, 1975; Meyer et al., 1998; Bell et al., 2002; Van Der Laan and Miles, 2010; Keeping et al., 2013). A third step is to augment and preserve soil moisture through practices that increase soil organic matter and improve rainwater infiltration (Thorburn et al., 1999; Bell et al., 2001; Pankhurst et al., 2005). Such measures, among others, will enable growers to minimize crop stress and re-adopt recommended N fertilization rates without the risk of economic losses to *E. saccharina* infestation.

AUTHOR CONTRIBUTIONS

Malcolm G. Keeping conceived and conducted the research, with advice and guidance on all plant nutritional aspects from Neil Miles. Trial designs and statistical analyses were performed by Chandini Sewpersad. The manuscript was written by Malcolm G. Keeping, and critiqued and approved by Neil Miles and Chandini Sewpersad.

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Leaf silica concentration in Serengeti grasses increases with watering but not clipping: insights from a common garden study and literature review

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Grasses (Poaceae) lack the complex biochemical pathways and structural defenses employed by other plant families; instead they deposit microscopic silica (SiO_2) granules in their leaf blades (i.e., phytoliths) as a putative defense strategy. Silica accumulation in grasses has generally been considered an inducible defense; other research suggests silica accumulation occurs by passive diffusion and should therefore be closely coupled with whole plant transpiration. We tested the hypothesis that grasses increase leaf silica concentration in response to artificial defoliation in a common garden study in the Serengeti ecosystem of East Africa. Additionally, a watering treatment tested the alternative hypothesis that leaf silica was largely driven by plant water status. Leaf silica content of two dominant C4 Serengeti grass species, *Themeda triandra* and *Digitaria macroblephara*, was quantified after a 10-month clipping × water experiment in which defoliation occurred approximately every 2 months and supplementary water was added every 2 weeks. *Themeda* had greater silica content than *Digitaria*, and *Themeda* also varied in foliar silica content according to collection site. Clipping had no significant effect on leaf silica in either species and watering significantly increased silica content of the dominant tall grass species, *Themeda*, but not the lawn species, *Digitaria*. Our data, and those collected as part of a supplementary literature review, suggest that silicon induction responses are contingent upon a combination of plant identity (i.e., species, genotype, life history limitations) and environmental factors (i.e., precipitation, soil nutrients, grazing intensity). Specifically, we propose that an interaction between plant functional type and water balance plays an especially important role in determining silica uptake and accumulation.

Keywords: grass, grazing, silica, defoliation, induced defense, herbivory, phytoliths

INTRODUCTION

Plants have two general and non-mutually exclusive adaptive strategies to cope with herbivory: tolerance and resistance (Mauricio et al., 1997). Tolerance implies the capability to survive and regrow following damage by herbivores. In contrast, resistance strategies serve to prevent or reduce damage by herbivores and may entail direct resistance via production of toxic or distasteful secondary metabolites or indirect resistance by avoiding herbivores altogether through reduced apparency (Feeny, 1976). From a resource allocation standpoint, defensive structures (i.e., spines, thorns) and phytochemicals (i.e., tannins) associated with direct herbivore resistance are costly because they require energy and nutrient resources that could otherwise be invested in growth or reproduction. Thus, while some defenses are constitutively expressed, many other plant defenses are induced only after damage is experienced as a way to reduce their costs (Agrawal and Rutter, 1998; Arimura et al., 2000; Massey et al., 2007).

Some plant groups face relatively intense or frequent herbivory and utilize both tolerance and direct resistance strategies. For example, many species of grasses (family *Poaceae*) experience herbivory in the form of defoliation by large-bodied mammalian grazers, resulting in frequent and significant tissue loss (Gibson, 2009). Due to rapid regrowth from a basal intercalary meristem following defoliation, grasses are highly resilient to grazing stress and are typically considered grazing "tolerators" (Stebbins, 1972; McNaughton, 1979; Oyarzabal and Oesterheld, 2009). On the other hand, grasses also utilize both secondary chemicals, e.g., phenolics (Schaller et al., 2012), and structural components, e.g., microscopic deposits of solid silica termed phytoliths (Ma and Yamaji, 2006), to deter herbivores. Evidence suggests that phytoliths have been present in grasses since their early evolution, as long ago as the Late Cretaceous (Prasad et al., 2005; Strömberg, 2011). Accumulation of silica phytoliths has been considered the main defensive strategy of grasses (Coughenour, 1985), as they can amass relatively large amounts of silica and lack chemical defenses as compared to dicotyledonous plants. However, other than grasses, silica accumulation occurs primarily in ancient plant groups such as mosses, ferns, and horsetail (Hodson et al., 2005), and this fact, along with the observation that tooth enamel is considerably harder than phytoliths (Sanson et al., 2007) raises questions about the efficacy of silica as a deterrent of large-bodied mammalian grazers. Among angiosperms, Poales (the group containing grasses) are the principal silica accumulators, with wetland Gramineae accumulating up to 15% dry weight silica (Epstein, 1999). Biogenic silica is assimilated when roots absorb silicic acid ($Si(OH)_4$) from soil water, and solid amorphous silica (SiO_2) bodies precipitate in target cells of the epidermis as transpiration occurs (see Rudall et al., 2014). Phytoliths leave behind a three-dimensional impression of the cells that they inhabit and, because of their diagnostic nature, are often used in paleoecological reconstruction (Piperno and Pearsall, 1998).

Both active and passive mechanisms for silica uptake have been documented within the plant kingdom; active exclusion has also been observed in dicotyledonous angiosperms (Jones and Handreck, 1969). Passive uptake allows grass roots to absorb silicon (Si) in its aqueous form, $Si(OH)_4$, from the soil solution and implies limited control over silica accumulation, with stomatal conductance largely determining foliar silica content (Sangster et al., 2001). While early researchers assumed that passive uptake, in which tissue silica concentration increases as a function of transpirational water loss (Sangster and Parry, 1970; Street, 1974), was the predominant mechanism for silica accumulation, several lines of evidence have since suggested that active uptake is also often involved. For example, specialized Si efflux transporters have been identified in rice (Ma et al., 2006, 2007), maize, and barley (Mitani et al., 2009) amongst other species, and gene expression of these transporters is positively correlated with Si absorption from the soil solution, implying an energetic cost associated with Si transport in these high-Si accumulators. Grass foliar silica content exhibits high intra- and interspecific variation (Hodson et al., 2005), often in ways that are correlated with disturbance regimes such as grazing and fire frequency (Melzer et al., 2009). These lines of evidence suggest that active Si transport may be an important, and prevalent, mechanism for silica accumulation within Poaceae. An active mechanism could prove especially beneficial if plants are able to respond to herbivory by increasing silica uptake and silica is an effective defense mechanism, as several studies have suggested. For example, high silica content in plant tissues interferes with digestion (Massey and Hartley, 2009), is unpalatable as forage (Gali-Muhtasib et al., 1992; Cotterill et al., 2007) and reduces growth rates of small-bodied mammals (Massey and Hartley, 2006). Consistent with the putative effects of silica on extant herbivores, paleontological research suggests that grazing Hadrosaurid dinosaurs evolved the most complex dentition known to date, at least in part due to a high silica diet (Erickson et al., 2012).

Inducible uptake of silica, via an active metabolic mechanism, may also prove advantageous for coping with several types of abiotic stress. In addition to its effects as a documented antiherbivore compound, biogenic silica is known to alleviate plant stressors such as heavy metals (Galvez et al., 1987), pathogenic pests (Fauteux et al., 2006; Heine et al., 2007), salinity (Zhu et al., 2004), high temperatures, and drought (Agarie et al., 1998). Thus, it is logical that uptake, if active, may also be inducible under particular environmental conditions. Water stress affects plants ubiquitously, and it appears that silica accumulation alleviates the deleterious effects of drought for grasses. Agricultural studies indicate that soil Si fertilization decreases stomatal conductance, and thus transpirational water loss (Street, 1974; Gao et al., 2006), and enhances the stability of rice cell membranes exposed to drought (Agarie et al., 1998).

The beneficial effects of plant biogenic silica, together with the apparently low cost of assimilating and using silica as a defense have left some to conclude that silica has largely been overlooked as a vital element for stress tolerance (Cooke and Leishman, 2011a). Our study had two goals relating to the ecological significance of plant silica. First, we aimed to quantify the response of leaf silica accumulation to interactions between water availability and defoliation in two African C4 grass species, one (*Themeda triandra*) a caespitose "bunch" grass and the other (*Digitaria macroblephara)* a short "lawn" grass. To achieve this goal we conducted a common garden study in Serengeti National Park, a grazer-dominated ecosystem in East Africa, in which we manipulated defoliation and water-availability over a 10-month growing period and quantified the silica responses of our two focal species. Previous research on similar grasses from the Serengeti demonstrated that grasses are capable of up-regulated silica concentrations in response to herbivory (McNaughton and Tarrants, 1983). Consequently, we hypothesized that defoliation would induce silica uptake in our two study species. In a different study of African savanna grasses, defoliated bunch grasses exhibited greater stomatal conductance and transpiration rates than defoliated lawn grasses (Anderson et al., 2013). Following these observations, we proposed a second prediction that, if silica uptake is directly linked to transpiration rate as previously reported, the bunch grass *T. triandra* would exhibit a greater silica induction following defoliation than would the lawn grass, *D. macroblephara.* Moreover, if silica accumulation is tightly coupled with transpiration rates, we expected to observe increased silica accumulation under conditions of higher soil moisture. Alternatively, if no relationship between soil moisture and silica concentration was observed, this suggests that active, energy-dependent silica uptake mechanisms may predominate in these species. Finally, we explored the possibility that the silica content of grasses was driven, at least in part, by their growing environment (soils, climate, herbivory, etc.). Thus, we also tested whether the site of collection was significantly related to silica variation among grasses in our common garden study.

Our second aim was to understand the results of our study in relation to a literature review of all studies reporting defoliation effects on leaf silica concentration in grasses. Our goal here was to search for a broad ecological consensus of graminoid responses to leaf defoliation and if possible, establish generalities about grass–grazer interactions and the induction of silica plant defense.

MATERIALS AND METHODS

COMMON GARDEN STUDY

Study system

Our study was conducted in the Serengeti ecosystem in northern Tanzania (2° 19′ 51′′ S, 34° 50′ 0′′ E). Serengeti is characterized by *Acacia-Commiphora* dominated savanna vegetation in the north and west of the ecosystem and edaphic grassland on volcanic soils in the Serengeti plains in the southeast (White, 1983). A rain-shadow created by the volcanic highlands to the south and east of Serengeti creates a relatively strong precipitation gradient, in which rainfall decreases from >1100 mm yr-1 in the northwest near the shores of Lake Victoria to ~600 mm yr-1 in the Serengeti plains at the base of the Ngorongoro crater in

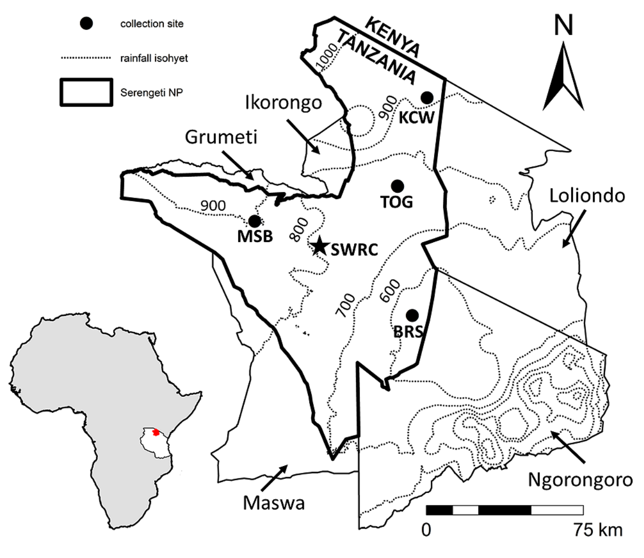

FIGURE 1 | Map showing the locations of the four collection sites of *Digitaria* and *Themeda* in Serengeti National Park; surrounding protected areas are directly labeled. Inset of Africa shows the location of this region in northern Tanzania in red. The common garden was constructed at the central Serengeti Wildlife Research Centre (SWRC). Mean annual precipitation is approximately 498 mm/yr at BRS, 676 mm/yr at TOG, 766 mm/yr at KCW, and 891 mm/yr at MSB (values from Anderson et al., 2007).

the southeast (**Figure 1**). Rainfall is highly seasonal and typically falls in two characteristic phases: the short rains, occurring from November–December, and the long rains, occurring from February–May (Anderson et al., 2007). The soils of the plains are heavily influenced by recent (~20,000 –1,500 ya) volcanic activity and are highly porous with an underlying calcium carbonate hardpan, resulting in highly saline and alkaline soils with poor moisture retention (Anderson and Talbot, 1965; de Wit, 1978). Interestingly, recent eruptions of nearby volcano Oldoinyo Lengai, which continue to enrich soils in the plains with ash, are of natrocarbonatite origin, meaning that, in addition to being highly enriched in potassium and sodium carbonates, they are extremely depleted of silica (Gittins, 1998; Keller et al., 2010). In contrast, the woodlands to the north are derived from granitic and quartzite parent material, while the western regions of the park are characterized by red clays (Ultisols) and black cotton soils (Vertisols) resulting from alluvial processes associated with Lake Victoria (Jager, 1982).

Serengeti is a “model” grazing ecosystem: massive herds of migratory zebra (*Equus quagga boehmi*) and wildebeest (*Connochaetes taurinus*) exert strong defoliation effects on the grasses that dominate the herbaceous layer. However, the frequency and intensity of herbivory changes along the rainfall gradient, with the most frequent and intense herbivory occurring in the Serengeti plains and decreasing with rainfall. Other significant herbivore species in Serengeti include Thomson’s gazelle (*Eudorcas thomsonii*), which also migrate, and several non-migratory herbivores such as Grant’s gazelle (*Nanger granti*), hartebeest (*Alcelaphus buselaphus*), and topi (*Damaliscus korrigum*). Running counter to the gradient in herbivory is an opposing gradient in fire frequency and intensity created by the tall, highly flammable, bunch grasses in the north and the procumbent, grazing tolerant grasses in the plains to the southeast that rarely burn due to their low aboveground biomass (Anderson et al., 2012). The two most dominant herbaceous species in Serengeti, in terms of both biomass and frequency, are the grasses *T. triandra*, a tall, caespitose bunch grass, and *D. macroblephara*, a short-stature, grazing tolerant lawn grass.

Experimental design

In of June 2008, multiple individuals of *T. triandra* and *D. macroblephara*, were collected from four grassland sites spanning the ecosystem’s soil and rainfall gradients and transplanted into a common garden constructed at the Serengeti Wildlife Research Centre (SWRC; **Figure 1**). *D. macroblephara* was collected from Barafu (BRS) in the southeastern plains, *T. triandra* was collected from Klein’s camp (KCW) in the northern corridor, and both species were collected from the Togoro plains (TOG) and Musabi plains (MSB). Grasses were planted in three replicate blocks; each block consisted of a 6 × 9 m fenced area cleared of all vegetation and included six 2 × 2 m equally spaced plots. Three randomly selected individuals of *T. triandra* and *D. macroblephara* (one from each collection site) were planted in each plot. Mean ± SE tiller number of initial *D. macroblephara* individuals was 13.59 ± 0.67, and *T. triandra* transplants had a mean ± SD = 9.08 ± 0.74 tillers. Plots were randomly assigned to one of the six combinations of clipping (two levels: clipped and unclipped) and watering (three levels: high, ambient and low). Water was manipulated by diverting rainfall with five 25 cm strips of clear plastic roof material placed across the plots at ~6 cm height and slanted slightly to promote water runoff; the treatments were designed to intercept approximately 50% of ambient rainfall. Grasses were planted between the roof strips and at least 45 cm from the nearest grass to provide room for growth and to prevent light competition. To control for any potential effects of the roofing material, all plots were overlaid by the roofing material but holes were drilled in the high and ambient treatments so that no water was diverted and all rainwater reached the plot. Rainfall was collected, and the high water treatment was augmented with an amount that totaled ~150% of ambient every 2 weeks. Rainfall at SWRC is approximately 700 mm yr^{-1} and the high water treatment was augmented to approximately 1000 mm yr^{-1}. Soil moisture at a depth of 12 cm was estimated via time-domain reflectometry monthly in each plot with a FieldScout TDR 100 (Spectrum Inc.; Aurora, IL, USA). In the clipping treatment, plants were defoliated to approximately 50% of their aboveground biomass every 2 months. Clipped biomass was dried, weighed, and summed with harvested biomass to arrive at a final biomass for clipped plants. Unclipped plants were not defoliated throughout the entire duration of the experiment. Plants were grown in the common garden for 10 months, until they were harvested in April 2009. All plants still alive at the harvest were separated into above- and belowground fractions; the aboveground fraction was further separated into leaf and stem. All plant material was dried and weighed in the lab at SWRC; leaf fractions were transported to the US, ground in a Cyclone sample mill (UDY corp.; Fort Collins, CO, USA) and stored until they were analyzed for leaf silica at Wake Forest University.

Biogenic silica quantification

Silicon content of leaf tissue was quantified by plasma spectroscopy (ICP-OES) after autoclave-induced digestion (AID) following the methods of Kraska and Breitenbeck (2010; modified from Elliott and Snyder, 1991). Briefly, 100 mg of dried, ground sample was wetted with 1-octanol in a 50 ml vortex tube. Next, 2 mL of 50% H_2O_2 and 3.5 mL of 50% NaOH were added. Samples were vortexed several times, until the reaction ceased, and autoclaved loosely capped at 121°C (20 psi) for one hour. Deionized water was added to 50 mL. Samples were brought to acidic pH using concentrated HCl, and diluted 1:10 in deionized water before analyzing by ICP-OES. Si content of samples was calculated by fitting peak intensity at 251 nm to a standard curve (0.1 –10 ppm Si; $r^2 \geq 0.998$). The standard curve was validated with a reference material of *Schizachyrium scoparium* (14 g/kg Si). Si values were converted to silica (SiO_2) content by dividing by a conversion factor of 0.4674, since this more commonly reported value represents biogenic silica (phytolith) content of plant tissue.

Statistical analysis

A major goal of our statistical analysis was to understand how defoliation, water addition and their interaction, influenced leaf silica concentration in these two dominant Serengeti grasses. Therefore, we used linear mixed effect (LME) models to test the effects of species, clipping, and water on foliar silica accumulation. The model included species, clipping, and water as fixed factors, while block was included as a random effect to account for the spatial design. Analysis was performed using the lmer function in *lme4* package of R statistical environment version 2.11.1 (Bates et al., 2014; R Development Core Team, 2014), with the original model structure: lmer(SiO2 ∼ species * clip * water + (1| block)), where "species*clip*water" represents the three-way fixed interaction effect and "(1| block)" represents the random intercepts that are estimated for each block. The function "step" from the R package *lmerTest* was used to simplify the model so that only significant fixed effects remained in the model, and pairwise contrasts for individual treatment effects were subsequently calculated using the "ghlt" command and a Tukey's test from the R package *multcomp* (Hothorn et al., 2008).

A second model was employed to explore the extent to which collection site could explain variation in foliar silica content for both species. Because we did not have sufficient replication of individual grasses at the site level, we could not explore the effects of site in a full model crossed with our other treatments. Consequently, we used a reduced statistical model to explore the main effects of site on leaf silica for each individual species averaged over all levels of water and defoliation. The model we employed for each species separately was: lmer(SiO2 ∼ site + (1| block)). Model simplification was again conducted via the "step" function in R.

Finally, soil moisture was statistically compared across treatments using a model with water as a fixed effect and time and block as random effects using the lmer function in *lme4* package of R as described above; the "ghlt" command from the R package *multcomp* was used to conduct a Tukey's *post hoc* comparison of means.

LITERATURE REVIEW

In order to identify general trends in plant response to defoliation, we conducted an extensive search of primary literature sources to identify studies which provided data on silica content of Poales under both control and defoliation treatments. We used Google Scholar and Web of Science to identify appropriate primary research articles by using combinations of search terms such as "clipping," "grazing," "defoliation," "silica," and "phytolith." Within the order Poales, defoliation studies were only identified for grasses, so the final literature review is limited to Poaceae. We restricted our search to studies containing species-specific silica values in order to avoid the potential confounding effects of differing community composition on bulk, plot level silica content. These studies included laboratory studies and field studies in which grazing was experimentally prevented by herbivore exclosures. Grazing studies encompassed insect, small mammal, and large mammal herbivory, and the intensity of defoliation varied within and among studies (see Discussion). Species-specific Si values were converted to SiO_2 when necessary, assigned a unique identifier, and plotted as the log ratio normalized difference between defoliated and non-defoliated plants at the species level. Studies were then organized by defoliation method and grazer type and assigned to a general defoliation response category to facilitate interpretation of the silica response. Those cases with a ≥ 20% increase in silica following defoliation were assigned a "+," those which decreased ≥20% were assigned a "−," and those exhibiting less than a 20% relative change in silica content were assigned a "0."

RESULTS

SERENGETI COMMON GARDEN

Mean ± SE soil moisture values were 11.1 ± 1.6% for the high, 9.8 ± 1.5% for the ambient and 9.7 ± 1.5% for the low watering treatment. *Post hoc* statistical comparisons with a Tukey's test demonstrated that the low and ambient water treatments were not statistically different from one another (difference = −0.1, $z = -0.26$, $p = 0.96$), but that the high water treatment was statistically greater than the low (difference = 1.4, $z = -2.9$, $p = 0.007$) and ambient (difference = 1.3, $z = 2.73$, $p = 0.01$) treatments. Therefore, the ambient and low water treatments are combined for the remainder of the manuscript (referred to as "ambient" from here onward) and compared to the high water treatment in all analyses of watering effects.

Both the bunch-grass *T. triandra* and lawn-grass *D. macroblephara* were relatively high Si-accumulators: *T. triandra* had a mean ± SE foliar SiO_2 content of 3.7 ± 0.25 % dw ($n = 20$), while *D. macroblephara* had a mean ± SE SiO_2 of 2.7 ± 0.10 % ($n = 41$). Neither species showed a significant response to clipping as a main effect ($p = 0.976$) or as an interaction effect when clipping was crossed with watering level ($p = 0.453$; **Figure 2**). However, there was a significant species by watering interaction effect ($p<0.05$, Supplementary **Table S1**) indicating that leaf silica concentrations of the two species differed in response to the watering treatment. This result arose because individuals of *T. triandra* that were watered had a higher leaf silica concentration (4.4 ± 0.3%) compared to those *T. triandra* plants that were maintained at ambient soil moisture levels (3.3 ± 0.3%). In contrast, no

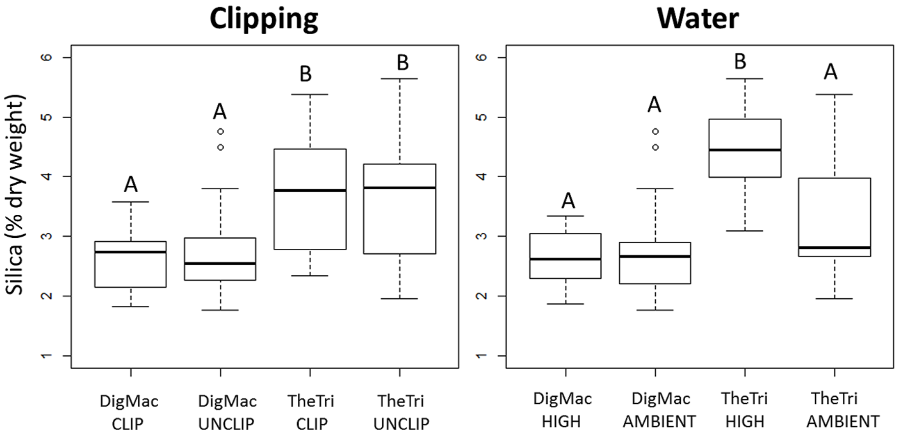

FIGURE 2 | Silica content of *Digitaria macroblephara* (DigMac) and *Themeda triandra* (TheTri) leaves as affected by defoliation and watering treatments. Defoliated individuals were clipped to 50% of maximum height bi-monthly a total of four times. High water treatment individuals received approximately 150% of ambient rainfall, and ambient treatment represents low and ambient water groups pooled (see Materials and Methods). Groups which differ significantly are indicated by a letter change.

significant change in SiO_2 content was observed between watering treatments for *D. macroblephara* (**Figure 2**).

In the second analysis, in which the statistical model included the main effects of plant collection site for each species separately, *D. macroblephara* exhibited considerable within-site variability and consequently no statistically significant differences across sites. In contrast, the SiO_2 content of *T. triandra* plants varied significantly among sites (**Figure 3**; see **Figure 1** for sites): Silica was lowest for plants collected from TOG (mean ± SE = 2.67 ± 0.63%), intermediate for plants from MSB (mean ± SE = 3.97 ± 0.55%), and highest for plants from KCW (mean ± SE = 4.18 ± 0.53%). Independent contrasts indicated that grasses from the low rainfall site TOG accumulated significantly less silica than those collected from MSB (difference = −1.30, $z = -2.822$, $p = 0.005$) and KCW (difference = −1.51, $z = -2.408$, $p = 0.016$).

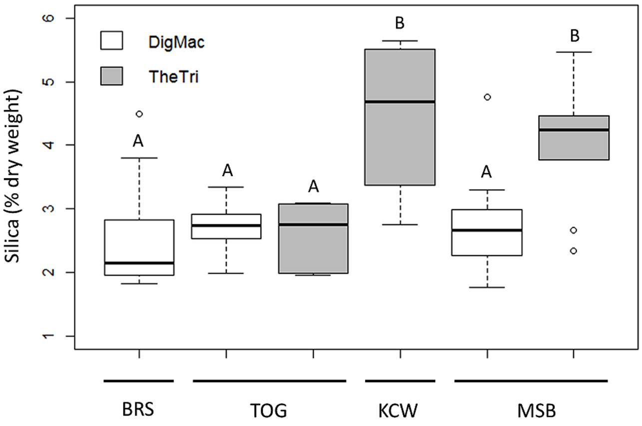

FIGURE 3 | Foliar silica content of each species in relation to plant collection sites. Both species were collected at the intermediate sites, MSB and TOG. *Themeda* (TheTri) does not occur at the shortgrass plains site BRS, and *Digitaria* (DigMac) does not occur at KCW in the northern woodlands. Sites are arranged in order of increasing mean annual precipitation: BRS = 498 mm/yr, TOG = 676 mm/yr, KCW = 766 mm/yr, MSB = 891 mm/yr.

LITERATURE REVIEW

We identified 11 studies conducted from 1974-present which recorded species-specific silica induction under control and defoliation treatments. These studies represented 34 "cases" of potential silica induction for 15 different grass species (Supplementary **Table S2**). Due to the limited number of studies conducted and lack of species-specific replication, we could not employ meta-analytical statistical approaches; instead, general responses were considered and interpreted. Eleven of fifteen species surveyed are C3, and all are perennials, except for *Poa annua.* Silica content of surveyed species ranged from <0.5% dry weight (non-defoliated *Anthoxanthum odoratum*) to >7% (grazed *Pascopyrum smithii*, *Deschampsia cespitosa,* and *Eustachys paspaloides*). To avoid pseudoreplication, we reported silica values for seasonal or site-level maxima for the three studies which reported multiple species-specific responses to the same defoliation method (Brizuela and Detling, 1986; Cid et al., 1990; Banuelos and Obeso, 2000). The majority of studies surveyed suggest that plants are able to respond to defoliation by altering their silica uptake, and indeed we observed an overall trend of silica induction following defoliation (**Figure 4**). Despite this general trend, several studies found no significant change in silica content of defoliated grasses, defined here as less than a 20% change in foliar silica content in either direction. The literature review revealed substantial variation in the silica responses of the different species studied. For example, the C3 grass *Festuca* increased silica content more than 350% in response to herbivory by voles (Massey et al., 2007), while silica content of *Pascopyrum* decreased by approximately 50% following clipping (Cid et al., 1990). In general, the magnitude of induced silica uptake was greater under natural defoliation (i.e., grazing) than for mechanical clipping. Further, the magnitude of silica increase when defoliation stimulated uptake

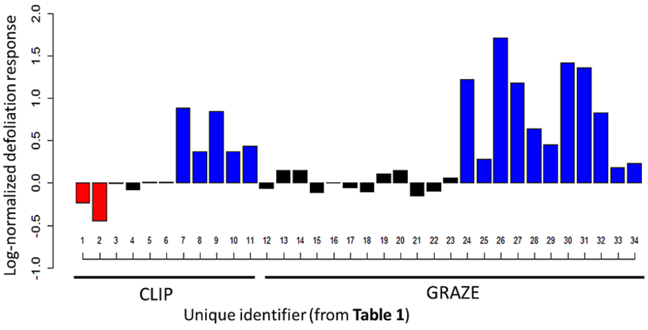

FIGURE 4 | Log-normal change in foliar silica content of defoliated grasses identified in the literature review. Blue bars indicate a ≥ 20% increase in silica following defoliation, red bars indicate a ≥ 20% decrease, and black bars indicate <20% change in either direction. Individual responses are arranged according to unique identifiers from Supplemenatry **Table S2**; Unique identifiers 1–11 represent clipping studies, and 12–34 represent grazing studies.

much greater than the decrease in silica levels observed in the two studies in which clipping triggered down regulation of silica levels.

DISCUSSION

VARIATION IN SILICA BETWEEN *Themeda* AND *Digitaria*

Both species documented in this study were within the typical silica range for dryland grasses, but *Themeda* had significantly greater silica content than *Digitaria*. These two species have different suites of anatomical and physiological traits and may be considered distinct graminoid functional types (Coughenour, 1985; Diaz and Cabido, 1997). Lawn grasses, such as *Digitaria*, exhibit clonal, stoloniferous growth; their prostrate growth form allows them to reduce tissue loss to herbivores while quickly spreading horizontally to form lawns under high soil fertility (Cromsigt and Olff, 2008). In contrast, the higher lignin content of tall, dense, and slow-growing bunch grass species, like *Themeda*, makes them less palatable to herbivores than lawn grasses (Sinclair and Norton-Griffiths, 1979). Thus, as a consequence of their different life history strategies, it is not surprising that these two species differ in the degree to which silica accumulates in their leaves.

As a documented growth promoter (McNaughton et al., 1985; Isa et al., 2010) and metabolically "cheap" structural substitute for carbon-based compounds such as lignin (Raven, 1983; Cooke and Leishman, 2011a), silica deposition may provide an alternative mechanism for accelerated growth which would prove especially beneficial for bunch grasses. The increased growth rates that result from silica accumulation may substantially improve light interception of slow-growing species which primarily compete for sunlight (Ando et al., 2002). This idea is further supported by a positive correlation between leaf length and silica content observed in *Spartina* (Querné et al., 2012), again supporting the idea that silica allows improved growth without requiring significant carbon investment (Cooke and Leishman, 2012). Further, slow-growing bunch grasses display greater leaf mass per area, LMA, (Fynn et al., 2011) and leaf dry matter content, LDMC, (Anderson et al., 2013) than fast-growing lawn species, indicating a greater relative investment in dry matter. The worldwide leaf economic spectrum (Wright et al., 2004) predicts a strong positive correlation between dry matter investment and leaf longevity, suggesting that, based on their higher LMA, bunchgrass species likely also have leaves of greater longevity than lawn-grass species. Thus, it may be beneficial for relatively long-lived bunch grasses to invest in immobile phytoliths (Endara and Coley, 2011) which allow for rapid augmentation of cell structure of C-limited species under intense light competition. On the other hand, recent research revealed a negative correlation between leaf lifespan and Si concentration when considering a broad range of plant functional types (Cooke and Leishman, 2011b). The reasons for differential responses to defoliation and high interspecific variation in this qualitatively ubiquitous defense within Poaceae remain unclear. Here we focused on two species representing extremes of each of the two grass growth forms (bunch, lawn); greater sampling from along the gradient of short to tall grasses is needed to better understand the influence of these contrasting physiological forms on silica accumulation.

DEFOLIATION RESPONSE

Contrary to our initial predictions, clipping did not result in silica induction for either the lawn or bunch grass species, with nearly identical mean silica content observed for defoliated and non-defoliated individuals. There are several possible explanations for the lack of silica induction observed in our Serengeti common garden study. First, the frequency and/or intensity of defoliation may not have been great enough to elicit a response. For example, in the Serengeti plains, more than 1 million migratory ungulates are present in dense herds during the wet season, and localized grazing "hotspots" support dense spatially and temporally stable grazer communities that inflict especially frequent and intense herbivory on plant communities (Holdo et al., 2009; Anderson et al., 2010). Our literature review further corroborates this notion. For example, a laboratory study by Massey et al. (2007) revealed that a single defoliation event did not induce silica uptake, while repeated defoliation by herbivores (16 events total) significantly increased silica content of ryegrass and fescue, revealing the importance of both frequency and duration of defoliation. Extent of tissue removal (5% vs. 25%) is also proven to affect both induction and relaxation of silica response, demonstrating a threshold effect required for induction (Reynolds et al., 2012). In our study, grasses were defoliated on a bi-monthly basis, four times total. For at least certain areas of Serengeti, this may be lower than levels of natural grazing intensity due to Serengeti's rich herbivore fauna.

In addition, manual defoliation (i.e., clipping) likely does not elicit the same response as natural grazing under laboratory or field conditions, a well-known effect with respect to induced defenses. Our literature review revealed that clipping and grazing both resulted in silica induction in ∼50% of the studies (5 of 11 clipping cases, 12 of 23 grazing cases), but the magnitude of this response was much greater under grazing defoliation, as illustrated in **Figure 4**. Among grazing studies, silica induction was observed in response to insect, small mammal, and large mammal grazing.

We propose that clipping may not be sufficient to represent both the direct and indirect effects of grazing on silica dynamics in grasses; this may be especially true for grass species growing in the Serengeti plains, which have a long co-evolutionary history with herbivores. Large-bodied grazers compact soils, resulting in increased bulk density (e.g., Bakker et al., 2004; Holdo and Mack, 2014; Veldhuis et al., 2014) and matric water potential and, thus, alter the ability of roots to absorb water. While manual defoliation studies yield information about certain physiological responses, the literature review presented here suggests that this approach may not properly mimic the complex physiological responses associated with natural grazing, some of which may result from indirect effects associated with modification of soil water (see below). In summary, plants perceive herbivore effects through a suite of signals, some of which, such as changes in the abiotic environment, are not elicited directly by the removal of tissues.

WATER AND COLLECTION SITE EFFECTS

Themeda individuals in the high water treatment accumulated significantly greater foliar silica than those in the ambient water group (**Figure 2**), but this trend was not observed for *Digitaria*. This suggests either differential silica uptake mechanisms of the two species or different generalized physiological responses to drought which indirectly influence silica accumulation. Since *Themeda* responded to changes in water availability, we hypothesize that active uptake mechanisms are present in this species which allow it to increase uptake of $Si(OH)_4$ from the soil solution when water is abundant. If true, this then raises the question, why would *Digitaria* not also increase uptake of a ubiquitously beneficial element under similar conditions? One possible explanation is that the different life histories of these two species, coupled with the proposed costs of silica accumulation (Cooke and Leishman, 2012) have resulted in selection for differential silica uptake mechanisms, in which tall, bunch species but not short, lawn species, have incorporated active Si uptake mechanisms such as the use of specialized energy-dependent transport molecules. *Digitaria* has highly flexible leaves which are capable of curling under drought conditions to prevent transpiration; this drought tolerance mechanism would likely be hindered due to the loss of bulliform cell function associated with leaf tissue silicification (Honaine and Osterrieth, 2011). Conversely, the benefit of improved drought tolerance associated with silica accumulation (Gao et al., 2006; Eneji et al., 2008) is likely of greater relative importance for bunch grass species, such as *Themeda*, which experience intense light competition and a significant seasonal water deficit. Increased silica uptake under high water availability may serve to buffer bunch grasses against future drought events while simultaneously producing an erect canopy structure (see Isa et al., 2010) for light competition and offsetting carbon costs. Under this proposed hypothesis, that bunch grasses benefit more than lawn grasses due to more intense light competition, any anti-herbivore benefits are secondary to the direct adaptive significance of silica accumulation, which is related to water and/or light limitation

Consistent with the observed effects of watering treatments, the silica differences among collection sites also indicate that water availability may be an important determinant of grass foliar silica content. *Themeda* individuals collected from high rainfall sites KCW and MSB, where light competition is expected to be more intense, exhibited significantly greater silica content than individuals collected from the low rainfall site TOG, where light competition is expected to be less intense. In contrast, the leaf silica content of *Digitaria* individuals was not influenced by collection site (**Figure 3**). This observation reinforces the notion that water strongly influences foliar silica content of grasses and implies a pattern of increasing silica content of tall bunch grass species, like *Themeda*, with increasing distance from the plains and increasing light competition.

Other possible explanations exist for the observed site-level variation in silica content of *T. triandra*. Numerous fertilization studies have indicated that plant silica content is strongly correlated with availability of $Si(OH)_4$ in the soil medium (Fox et al., 1969; Jones and Handreck, 1969; Van der Vorm, 1980; Gali and Smith, 1992), and more recently, ecological studies have demonstrated a similar relationship between soils and plants in natural systems. Biogenic silica content of grasses in a South African savanna was higher for plants collected from basaltic soils than for those collected from granitic soils, reflecting differences in weatherability and dissolved silica (DSi) content of these contrasting parent materials (Melzer et al., 2011). Cooke and Leishman (2012) compared foliar silica content of plant communities from Hawkesbury sandstone and a nearby diatreme forest with nearly three times as much plant available soil Si; though not statistically significant, they observed a general trend of higher foliar silica content for plants from the diatreme site, including a fivefold difference in leaf silica observed for the sedge *Schoenus melanostachys*. The unique nature of the silica-depleted carbonatite ash that enriches the Serengeti plains may contribute to a gradient in available soil Si, in which available Si increases toward the northern and western corridors of the park. However, more work is needed to document how soil Si availability varies within SNP and how it may interact with gradients in soil moisture, pH, and other important soil nutrients known to vary across the landscape (de Wit, 1978; Jager, 1982). Finally, we caution that, due to geographic separation, plant genotype should also vary among sites (Soininen et al., 2013), and we cannot rule out genotypic variation as an important influence on silica accumulation patterns.

ECOLOGICAL AND EVOLUTIONARY IMPLICATIONS

The results of our literature review suggest that induced silica response to defoliation is dynamic and dependent upon interactions with additional biotic and abiotic factors. For example, defoliation by burning resulted in more than a 1/3 decrease in silica content of *Aristida* grass (Dufek et al., 2014). Moreover, seasonal (Georgiadis and McNaughton, 1988, 1990; Carey and Fulweiler, 2013; Struyf et al., 2005) and inter-annual (Morton and Jutras, 1974; Pahkala and Pihala, 2000) variations in field measurements of grass silica content indicate that leaf tissue silicification is highly plastic. While seasonal fluctuations in grass silica content are well-documented, it remains unclear whether dynamic silica values measured in the field occur in response to changes in grazing intensity, temperature, precipitation, or, most likely, a complex interaction among these stochastic variables.

Silica accumulation is considered a central axis of grass–grazer coevolution throughout the literature, often cited as a driving force behind the evolution of hypsodonty and the coupled taxonomic radiation of grasses and grazers (Mendoza and Palmqvist, 2008; Hummel et al., 2011). The evolution of large-bodied mammalian grazers is thought to have selected for specialized traits of the Poaceae such as meristematic growth from a well-protected crown, rapid growth rate, and high levels of Si accumulation (McNaughton, 1979; Coughenour, 1985). Likewise, these plant traits are thought to have influenced herbivore dentition, digestion, and behavior (McNaughton, 1984; Massey and Hartley, 2006; Erickson et al., 2012). Our study and literature review, however, suggest that the evolutionary significance of silica accumulation may be a remnant of tradeoffs associated with the leaf economic spectrum that resulted in the evolution of two contrasting grass functional types. Silica accumulation may have first served to improve drought tolerance with herbivore deterrence as a secondary role. Fossil data support this notion: grass-dominated habitats preceded the appearance of North American and Mediterranean grazing specialists (Strömberg, 2006; Strömberg et al., 2007), and an investigation of the Gran Barranca fossil record indicates that open grass habitats were not necessary pre-conditions for favoring early hypsodont mammals in Earth's earliest grass-dominated systems (Strömberg et al., 2013).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00568/abstract

Table S1 | Results from linear mixed model in R testing for interactions between species and water on foliar silica accumulation. Block was included as a random effect. *P* values in bold indicate significance at $\alpha = 0.05$.

Table S2 | Summary of studies included in literature review of induced silica response of Poaceae; colors in the "Defoliation Response" column correspond with Figure 4. In the *Defoliation Response* column, "0" indicates that a species exhibited less than a 20% relative change in foliar silica content in response to defoliation, while "+" indicates a $\geq$ 20% increase in silica, and a "−" indicates a $\geq$ 20% decrease in silica.

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Defending the leaf surface: intra- and inter-specific differences in silicon deposition in grasses in response to damage and silicon supply

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Understanding interactions between grasses and their herbivores is central to the conservation of species-rich grasslands and the protection of our most important crops against pests. Grasses employ a range of defenses against their natural enemies; silicon-based defenses have been shown to be one of the most effective. Silicon (Si) is laid down on the leaf surface as spines and other sharp bodies, known as phytoliths, making grasses abrasive and their foliage indigestible to herbivores. Previous studies on Si defenses found that closely related species may have similar levels of Si in the leaves but differ markedly in abrasiveness. Here we show how the number, shape and distribution of Si-rich phytoliths and spines differ within and between different grass species and demonstrate that species also differ in their ability to change the deposition and distribution of these defenses in response to damage or increases in Si supply. Specifically, we tested the response of two genotypes of *Festuca arundinacea* known to differ in their surface texture and three different grass species (*F. ovina*, *F. rubra*, and *Deschampsia cespitosa*) differing in their abrasiveness to combined manipulation of leaf damage and Si supply. *F. arundinacea* plants with a harsh leaf surface had higher Si content and more spines on their leaf surface than soft varieties. *F. ovina* and *D. cespitosa* plants increased their leaf Si concentration and produced an increase in the number of leaf spines and phytoliths on the leaf surface in response to Si addition. *F rubra* also increased leaf Si content in response to treatments, particularly in damaged leaves, but did not deposit this in the form of spines or increased densities of phytoliths. We discuss how the form in which grasses deposit Si may affect their anti-herbivore characteristics and consider the ecological and agricultural implications of the differences in allocation to Si-based defenses between grass species.

Keywords: plant defense, silicon, abrasion, grasses, leaf surface, phytoliths, spines

INTRODUCTION

Grasslands including managed rangelands and pastures cover ~40% of the earth's surface and grasses are an important plant family agriculturally, economically and ecologically (Strömberg, 2005; Gibson, 2009). Not only are our most widely grown and consumed food crops domesticated grass species, but grasses also provide grazing for both wild and domesticated animals. In their long co-evolution with grazers (Coughenour et al., 1985), grasses have developed a number of defensive strategies to both tolerate and repel herbivory (Vicari and Bazely, 1993), including rapid regrowth ability from their basal meristems (also an adaptation to fire and trampling common in these ecosystems) and a combination of both chemical defenses (including those provided by endophyte mutualists; Hartley and Gange, 2009) and physical defenses (McNaughton and Tarrants, 1983).

One such physical defense is the accumulation of silicon (Si) which has been previously reported to accumulate in high levels in the leaves of many grass species (Hodson et al., 2005), although the amount of Si accumulated shows large inter and intra species variation (Massey et al., 2007a; Soininen et al., 2013). There is clear evidence to demonstrate that these high levels of Si are effective anti-herbivore defenses, with impacts on the feeding preferences and performance of both vertebrate (McNaughton and Tarrants, 1983; Gali-Muhtasib et al., 1992; Massey and Hartley, 2006; Teaford et al., 2006; Massey et al., 2009) and invertebrate herbivores (Goussain et al., 2005; Massey et al., 2006; Kvedaras et al., 2007; Massey and Hartley, 2009).

These adverse effects appear to be mediated at least in part by abrasion: Si is primarily deposited as amorphous silica in the form of solid bodies known phytoliths in the epidermis (Richmond and Sussman, 2003; Currie and Perry, 2007). Phytoliths are hard and often irregular shapes and Si is also deposited in leaf hairs, trichomes and spines; all these structures could influence the texture and abrasiveness of the leaf. It has been suggested that Si abrades the teeth of mammalian herbivores (Jernvall and Fortelius, 2002; Erickson, 2013 but see Sanson et al., 2007) and an increase in leaf abrasiveness has been shown to reduce the performance of both vertebrate and invertebrate herbivores. For example, the amount of mandible wear feeding imposed on African armyworm (*Spodoptera exempta* Walker), and hence the reduction in their ability to extract nitrogen from their food, is correlated with the Si levels of the foliage they consume (Massey and Hartley, 2009), whilst voles prefer, and perform better on, grasses which are less abrasive (Massey and Hartley, 2006; Massey et al., 2008).

Previous work has suggested that foliar Si levels and the abrasiveness of grass leaves are reasonably well correlated: over 70% of the variation in abrasiveness across 18 different grass species was explained by Si content (Massey et al., 2007a). However, Si levels and abrasion are not always closely linked. For example, despite containing similar concentrations of Si, *Festuca ovina* L. was found to have much higher levels of abrasiveness compared to *F. rubra* L., whilst increasing leaf Si concentration through Si addition produced a smaller increase in abrasiveness in *Poa annua* L., a relatively palatable species, than in the more unpalatable *Brachypodium pinnatum* (L.) P. Beauv. (Massey et al., 2007a). It is possible that different grass species deposit their available Si differently at their leaf surfaces, influencing the abrasiveness of their leaves. It is certainly well-known that phytolith morphology varies between plant taxa, with differences between species sufficiently marked and consistent to allow phytoliths to be useful in palaeobotany (Strömberg, 2005). Some phytoliths are relatively smooth in shape, others much less so and it seems likely that the size, shape and density of phytoliths and Si rich spines will influence the abrasiveness of the leaf surface and its impact on the preferences and performance of herbivores.

Another influence on the nature and effectiveness of the leaf surface defenses will be the amount of Si available in the soil to take up and deposit (Currie and Perry, 2007). Previous exposure to herbivory has also been shown to impact on the levels of Si-based defenses in plants. It has long been known that Si levels increase in grasses from grazed areas (McNaughton and Tarrants, 1983) and herbivore-specific induction in Si defenses has been shown to occur, but only after repeated damage above a threshold (Massey et al., 2007b; Reynolds et al., 2012). More recently it has been shown that there are differences in both grass species and grass genotypes in the extent to which they respond to damage with increased Si uptake (Soininen et al., 2013).

The aim of this study was to determine the leaf Si concentration of different forage grass genotypes and naturally occurring grass species previously reported to differ in their leaf abrasiveness (see below), and to investigate whether these differences in leaf texture are related to the way Si is deposited on the leaf surface, potentially influencing the effectiveness of their use of Si in terms of reducing palatability to herbivores. We hypothesized that:

(i) harsher and more abrasive species and varieties would have higher leaf Si levels than softer ones;
(ii) species with similar Si levels which differed in abrasiveness would do so because they used their Si to produce a greater number of phytoliths and/or spines on their leaf surface, and that these spines would be larger or sharper.

We also hypothesized that irrespective of grass species, foliar Si levels would be elevated by increases in Si supply, and hence in potential uptake (Epstein, 1999; Cooke and Leishman, 2011), and by damage, due to induction (Massey et al., 2007b). We also expected foliar Si levels to be highest in plants receiving both Si addition and damage, since induction in response to damage would be able to capitalize on the additional Si available in the soil. We predicted that the most abrasive species would deposit this additional Si in the form of surface spines to a greater extent than less abrasive species.

MATERIALS AND METHODS

STUDY SPECIES

Festuca arundinacea Schreb. is a cool season perennial grass (Gibson and Newman, 2001) and a dominant pasture and turf grass in North America, Australia and Europe (Hand et al., 2012). It has a number of attractive agronomic attributes, including high yields, winter persistence (Gibson and Newman, 2001) and tolerance to drought (Cougnon et al., 2014), though it appears to be relatively unpalatable to cattle. In mixed culture fields, cattle rarely choose it as their forage choice (Gibson and Newman, 2001), possibly because of the "harsh" (i.e., feeling rough to the touch) texture of the leaf surfaces. There is interest amongst forage breeders in understanding the basis of this leaf harshness and unpalatability to improve the attractiveness of this species as forage. A number of varieties of *F. arundinacea* ranging from very harsh to very soft leaf textures have been developed by plant breeders (based on manual evaluation of surface roughness in the field by plant breeders), enabling the testing of the hypothesis that Si has a role in causing the harsh leaf surfaces.

We can also address the relationship between Si content and leaf texture by exploiting the natural variation in the relationship between Si and abrasion across native non-forage *Festuca* species: *F. ovina* and *F. rubra* may differ so markedly in their leaf abrasion despite similar foliar Si levels (Massey et al., 2007a) because of the way they utilize the Si they take up. Specifically, *F. ovina* may produce a greater number, larger or more abrasive spines and phytoliths than *F. rubra*. We compared these species with the Si content and leaf texture of *Deschampsia cespitosa*, a grass known to be particularly unpalatable to herbivores due to its Si defenses (Massey and Hartley, 2006, 2009).

PLANT GROWTH AND EXPERIMENTAL TREATMENTS

The two varieties of *Fesctuca arundinacea* were grown individually from seed in a loam based compost (John Innes No.2) in 13 cm pots. Both varieties were harvested 8 weeks after sowing at the point where all plants had at least four tillers. *F. ovina, F. rubra*, and *D. cespitosa* (L.) were grown from seed individually in peat based F2 (Levington, Scotts) compost in 10 cm diameter pots in the greenhouse conditions 16 h daylight, 20°C/15°C day/night. Due to their slower growth rates in relation to *F. arundinacea*, these three species were harvested 18 weeks after sowing.

Once established, plants were randomly assigned to four treatments: control plants with no Si addition and no damage (Si- D-), Si addition only (Si+ D-), damage only (Si- D+), and both Si addition and damage (Si+ D+). Treatments were imposed 3 weeks after sowing in the case of *F. arundinacea*, with plants harvested 5 weeks later, and 8 weeks after sowing in the case of the other three species, which were harvested 10 weeks later. There were six replicate plants of each treatment combination for *F. arundinacea* and seven replicate plants of each treatment for the other species.

For all grass species and varieties, Si addition was achieved by watering plants with 150 mg L^{-1} solution of dissolved sodium metasilicate ($Na_2SiO_3 \cdot 9H_2O$). Plants were watered 100 ml twice a week with either Si solution or deionised water. *F. ovina, F. rubra*, and *D. cespitosa* plants in the two treatments where damage was applied were mechanically damaged using scissors once a week over 10 weeks. Half of the plant's leaves were damaged by removing approximately half the leaf lamina down the midrib; the remaining leaves were left undamaged. When damaged plants were harvested, damaged and undamaged leaves were kept separated for Si analysis in order to test for induction of Si defenses in both the undamaged and the damaged leaves on the damaged plants.

Si ANALYSIS BY PORTABLE X-RAY FLUORESCENCE SPECTROMETRY (P-XRF)

Silicon was analyzed by P-XRF, calibrated using Si-spiked synthetic methyl cellulose and validated using Certified Reference Materials of NCS ZC73014 'Tea' obtained from China National Analysis Center for Iron and Steel (Reidinger et al., 2012).

Both P-XRF and EDX (see below) work on the principle of excitation of inner orbital electrons by an X-ray radiation source. As the excited electrons relax to the ground state, they fluoresce, thereby ejecting photons of energy and wavelength characteristic of the elements present and their concentrations. XRF instruments are widely used for the non-destructive, rapid and accurate elemental analysis of a range of materials (Jang, 2010).

Leaf material was ball milled (Retsch MM 400, Haan, Germany) for 2 min at a vibrational frequency of 24 Hz (60 min^{-1}) with two 1 cm diameter steel grinding balls in a 25 ml grinding jar. Leaf material was pressed at 11 tons for approximately 5 s into 5 mm thick cylindrical pellets with a manual hydraulic press using a 13 mm die (Specac, Orpington, UK). Si analysis (% Si DW) was performed using a commercial P-XRF instrument (Nitron XL3t900 GOLDD analyser: Thermo Scientific Winchester, UK) held in a test stand (SmartStand, Thermo Scientific, Winchester, UK).

SURFACE ANALYSIS BY SCANNING ELECTRON MICROSCOPY (SEM) AND ENERGY DISPERSIVE X-RAY SPECTROSCOPY (EDX)

Leaf samples were taken from two replicate plants per species from all four treatment combinations for the inter-species experiment. A square section (~5 mm^2) of leaf material either side of the midrib of a mature, expanded leaf blade on the main stem was cut with a razor blade and immediately placed in fixative (2.5% glutaraldehyde, 4% formaldehyde in 100 mM phosphate buffer). For the *F. arundinacea* experiment, samples were taken from the harsh variety with added Si and the soft variety with no added Si (Si addition had no effect on Si levels in this experiment – see below). A square section (~1 cm^2) spanning the entire width of the mature, expanded leaf blade was cut from the main stem for each variety. The samples were then dehydrated through an acetone graduated series (samples were placed at 25, 50, 75, and 100% acetone concentration for ~1 h) and critical-point dried. Samples were then mounted on sticky carbon tabs and coated with 8nm thick layer of platinum-palladium.

SEM images were obtained using FEI Sirion S-FEG FESEM (Oxford Instruments, Tubney Woods, Abingdon, Oxfordshire). EDX was used in conjunction with the SEM to determine the elemental composition of the samples; an electron beam was focused on the samples and the difference between the ground state (unexcited state) and the excited state was measured by the energy-dispersive spectrometer which determines the elements present in the sample (Goldstein, 2003). The EDX analysis was performed using an Oxford INCA analysis system FESEM (Oxford Instruments, Tubney Woods, Abingdon, Oxfordshire), using the working distance of 10 mm. For the SEM images, the voltage was 5–10 kV and for the EDX analysis the voltage was 12 kV.

STATISTICAL ANALYSES

All analyses were performed using R (version 3.0.2). ANOVA was used to test the main and interactive effects of grass species or genotype, Si addition and damage treatments on leaf Si concentrations. The effects of the Si and damage treatments were assessed on undamaged leaves from plants across all four treatments, to test if damage led to increased Si levels systemically in damaged plants in comparison with undamaged plants. A separate analysis tested for the effect of these treatments in damaged leaves from damaged plants compared to undamaged leaves from undamaged plants. *Post hoc* Tukey contrast tests were performed using the ghlt function from multcomp package (Hothorn et al., 2014).

Linear models were used to check for normality and homogeneity of variance following Crawley (2007). Si (%) values were transformed using the arcsine squareroot transformation to meet the assumptions of the test. Significance was set at $P < 0.05$ for all analyses.

RESULTS

INTRASPECIFIC DIFFERENCES

The *F. arundinacea* variety with a harsh leaf surface texture had significantly higher leaf Si concentration than the soft texture variety ($F_{1,19} = 8.586$, $P < 0.01$), but there was no significant interaction between Si addition and variety ($F_{1,19} = 0.282$, $P > 0.5$; **Figure 1**).

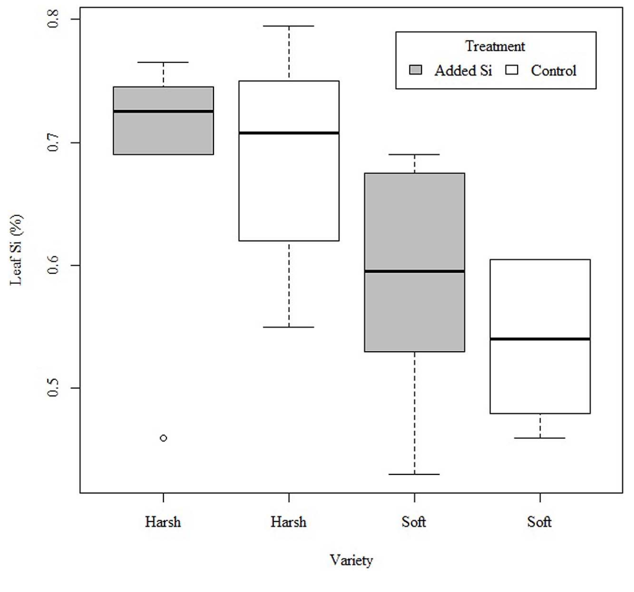

FIGURE 1 | Silicon (Si) concentration (%) of harsh and soft variety of *Festuca arundinacea* with no Si addition (control) or Si addition (+Si).

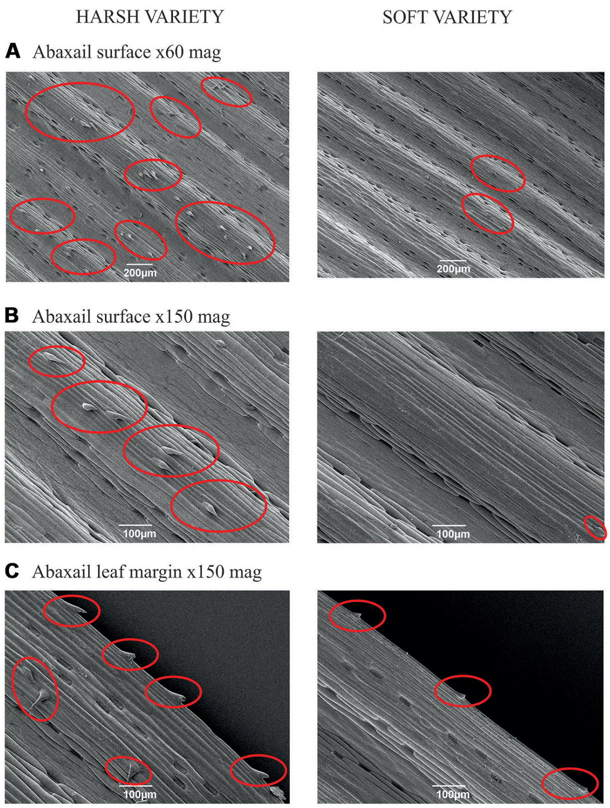

FIGURE 2 | (A) Harsh and soft variety abaxail surface (x60 magnification), **(B)** Harsh and soft variety abaxail surface (x150 magnification), **(C)** Harsh and soft variety abaxail margin (x150 magnification). Red circles indicate leaf spine presence.

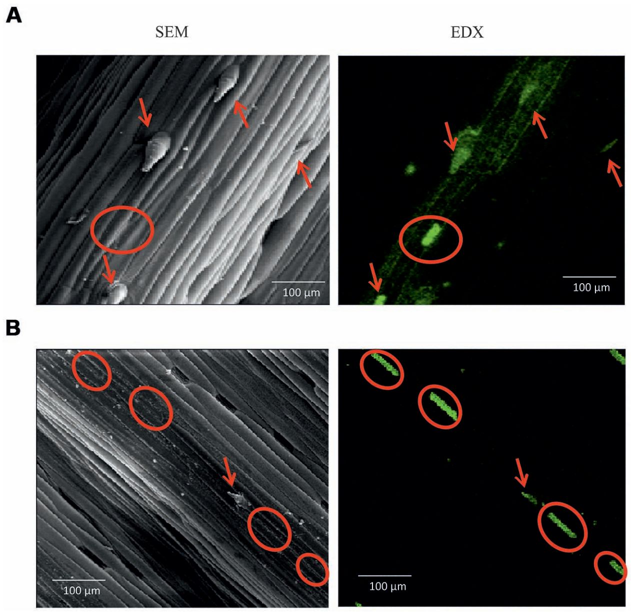

FIGURE 3 | (A) Harsh variety abaxail surface (x200 magnification), **(B)** Soft variety abaxail surface (x200 magnification). Scanning electron microscope (SEM) represented by grey images. Electron density X-ray spectroscopy (EDX) represented by black and green images; green intensity indicates Si concentration. Red circles indicate trichomes with Si deposition. Red arrows indicate silica short cells.

The two main types of cells which were silicified were leaf spines (trichomes) and silica short cells. The harsh variety had more spines present on the abaxial surface than the soft variety (**Figures 2A,B**), which not only had fewer spines but the spines which were present were smaller and had a different morphology (**Figures 2B,C**). The spines present on the harsh variety were bigger in size and the point of the spines were spear-like in appearance; these spines also appeared to protrude more from the surface compared with the soft variety, where the spines were smaller in size and the points of the spines lay closer to the surface of the leaf. No spines were observed on the adaxial surface of either variety of *F. arundinacea* (images not shown).

The spines were rich in Si (**Figure 3**), and there were other Si deposits on the leaf surface in the form of silica short cells. Generally, the harsh variety had a greater over surface deposition of Si compared with the soft one, depositing the Si within the leaf spines (red circles **Figure 3**), and also silica short cells surrounding the spines (red circles **Figure 3**). The soft variety deposited Si as silica short cells on the leaf surface within fewer, smaller leaf spines containing less Si than in the harsh variety (**Figure 3B**).

INTERSPECIFIC DIFFERENCES

Grass species differed in their leaf Si concentration (**Figure 4**; Spp effect: $F_{2,72} = 23.62$, $P < 0.001$ undamaged leaves; $F_{2,72} = 15.99$, $P < 0.001$ damaged leaves) with significantly lower Si concentrations in *D. cespitosa* compared to *F. rubra* (*post hoc* Tukey tests $P < 0.05$ for both undamaged and damaged leaves) and *F. ovina* (*post hoc* Tukey tests $P < 0.01$ for both undamaged and damaged leaves). Plants treated with Si addition responded with an increase in their leaf Si concentration irrespective of whether leaves were damaged or not (Si effect $F_{1,72} = 1265.33$, $P < 0.001$ undamaged leaves; $F_{1,72} = 463.17$, $P < 0.001$ damaged leaves). In

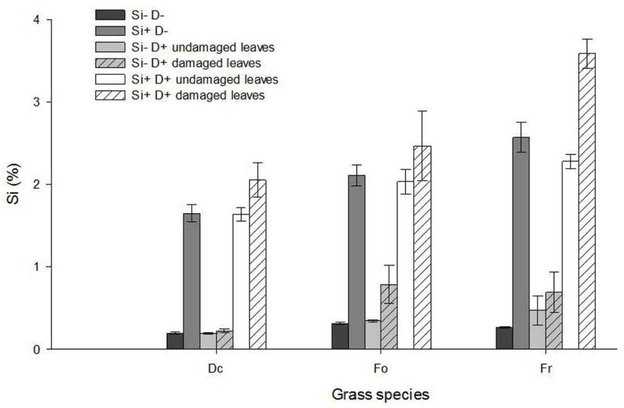

FIGURE 4 | Leaf Si concentration (%) of *D. cespitosa* (Dc), *F. ovina* (Fo) and *F. rubra* (Fr) plants treated with no Si addition and no damage (Si- D-), Si addition only (Si+ D-), damage only (Si- D+), and both Si addition and damage (Si+ D+). Values represent mean ± SE bars of seven replicates.

comparison with undamaged leaves on undamaged plants, damage did not increase Si levels in undamaged leaves on damaged plants ($F_{1,72} = 0.03$, $P > 0.05$ NS), but there was a significant increase in the Si levels in the damaged leaves ($F_{1,72} = 17.92$, $P < 0.001$), suggesting that damage-induced increases in Si levels are localized in damaged leaves and do not spread to undamaged ones on the same plant (**Figure 4**).

In the case of undamaged leaves there was no significant interaction between the effects of species and Si addition on Si levels ($F_{2,72} = 2.89$, $P > 0.05$ NS), suggesting all three species responded in a similar way to increases in Si supply in terms of the allocation of this additional Si to their undamaged leaves. However this was not the case for damaged leaves, where a significant Species × Si addition interaction ($F_{1,72} = 4.62$, $P < 0.05$) suggests species differ in where they allocate any additional Si once they are damaged. This is confirmed by the *post hoc* Tukey tests which showed that *F. rubra* had significantly higher concentrations of Si in damaged leaves under conditions of increased Si supply than either *F. ovina* ($P < 0.01$) or *D. cespitosa* ($P < 0.001$; **Figure 4**).

The SEM revealed differences in Si deposition on the leaf surfaces of the grass species (**Figure 5**). The leaf surface of *D. cespitosa* plants was found to have abundant Si-rich leaf spines (trichomes), even in the absence of Si addition. In contrast, the leaf surface of *F. rubra* and *F. ovina* plants growing without added Si had only rounded silica short cells and no leaf spines, although the round phytoliths were much more prominent and frequently distributed on the leaf surface of *F. ovina* than *F. rubra*. Both *F. ovina* and *D. cespitosa* plants deposited additional phytoliths (silica short cells) in response to increased Si supply, especially in the presence of damage, but Si addition had very little effect on the number or shape of the phytoliths deposited on the leaf surface of *F. rubra*. The damage alone treatments had little effect on leaf surface Si deposition in any of the grass species (images not shown).

DISCUSSION

Grass species and varieties differed in their leaf Si concentrations and the form in which this Si is deposited on the leaf surface. The more unpalatable and abrasive species, namely *D. cespitosa* and the harsh variety of *F. arundinacea* had both larger and a greater abundance of Si-rich spines compared to the more palatable *F. rubra* and soft variety of *F. arundinacea*. Si addition resulted in an increase in leaf Si concentration in three out of the four grass species and altered the deposition of Si on the leaf surface in the case of *D. cespitosa* and *F. ovina*, but had little impact on the surface of *F. rubra*. The different forms in which Si is deposited at the leaf surface in *F. ovina* and *F. rubra* may explain previous observations that they differ in abrasiveness even though, as we found here, they contain similar amounts of Si. Generally, damage caused a small increase in leaf Si concentration, but did not have a large effect on the form in which the Si was deposited on the leaf surface.

INTRASPECIFIC DIFFERENCES

As we hypothesized, the harsh variety of *F. arundinacea* had a higher leaf Si level than the soft variety, though our prediction

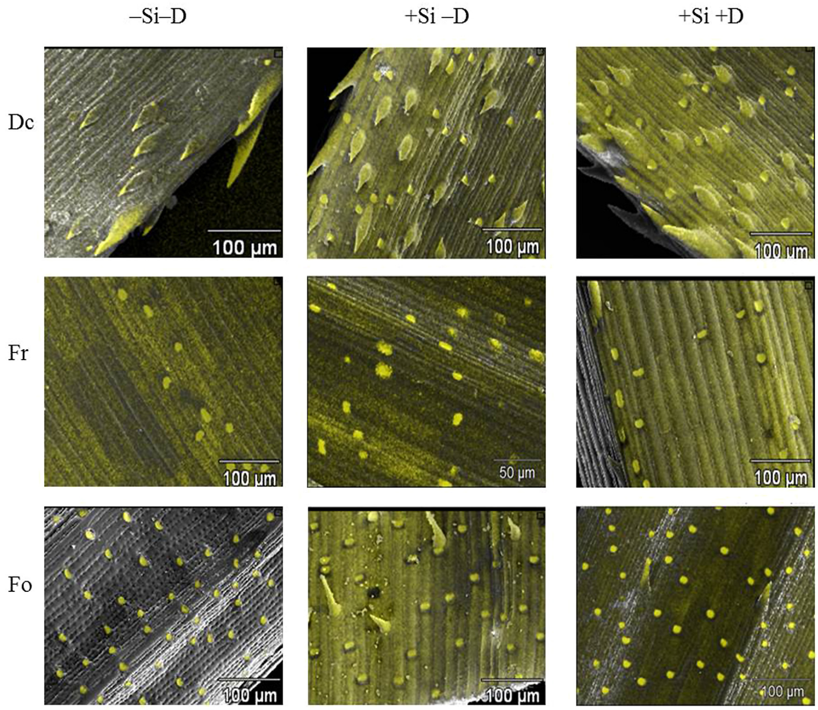

FIGURE 5 | Images (x300 magnification) of *D. cespitosa* (Dc), *F. ovina* (Fo), and *F. rubra* (Fr) plants treated with control (Si- D-), Si addition with no damage (Si+ D-) and Si addition and damage (Si+ D+). Yellow intensity indicates Si concentration. Red circles indicate trichomes with Si deposition. Red arrows indicate silica short cells.

that Si addition would increase foliar Si content was not supported in this species. The differences in foliar Si content between the harsh and soft varieties suggest that Si is contributing significantly toward the differences in leaf texture between them. The harsh variety had significantly higher leaf Si content than the soft variety as well as having a different leaf surface morphology, suggesting that increased levels of Si support the production of increased number and size of leaf spines. This is significant in the context of forage grass: differences in the patterns of deposition of Si on the leaf surface between varieties may offer scope for plant breeders to select for more palatable forage.

Scanning electron microscopy images also revealed that the harsh variety of *F. arundinacea* had more numerous and larger leaf spines than those on the leaf surface of the soft variety, and this was particularly noticeable at the leaf margin. These spines may act as a deterrent to herbivores, especially cattle as they use their tongues to wrap around the blades of grass prior to chewing; if the grass feels spiny then it is likely to seem unpalatable to the cattle. An impact of Si levels on the bite rate of ruminants has been demonstrated experimentally for sheep (Massey et al., 2009), suggesting Si does impair food processing time. The soft variety had far fewer and smaller spines, suggesting these plants are depositing Si in a different way to the harsh variety. Even when not deposited as abrasive spines, Si can still make plants hard to digest, if for example these deposits prevent herbivores crushing cells to extract nutrients, as hypothesized by Hunt et al. (2008).

Contrary to our predictions, neither variety responded to the Si addition treatment with an increase in leaf Si content. This may be related to the young age of the plants and relatively short duration of the Si addition treatment. Si accumulation is influenced by transpiration rates, where older leaves are found to have significantly more Si than younger leaves, primarily due to Si translocation via the transpiration stream (Piperno, 2006). Furthermore, once deposited Si is not remobilized (Richmond and Sussman, 2003), meaning foliar Si levels increase with both plant and leaf age (Reynolds et al., 2012). Although the mechanisms underpinning Si uptake and distribution in plants are still not fully understood, it has been demonstrated that plant species differ in the Si uptake ability of their roots and in the density of their root transporters, as well as in their capacity to upload Si to the xylem (Ma and Yamaji, 2006). More recently, work on rice has suggested that shoots control the regulation of the Si transporters in the root and hence how much Si is taken up into the shoot (Yamaji and Ma, 2011). A study assessing the uptake abilities of over 500 plant species (Ma and Takahashi, 2002) classified them into high, intermediate and non-accumulators and it may be that *F. arundinacea* physiology is such that it is not a high accumulator of Si, even under conditions of high Si supply. It does however, appear to be able to use the Si it does take up very efficiently in terms of spine production, at least in the case of the harsh variety. The mean Si values reported here for *F. arundinacea* are lower than those reported for this species in Hodson et al. (2005), which may reflect differences in age of the plants when sampled or the growing conditions of the plants. However the variation in foliar Si content shown in this species and indeed in other taxa within the genus (Hodson et al., 2005; Massey and Hartley, 2006; Massey et al., 2006, 2007a, 2009) suggests a high degree of phenotypic plasticity in the levels of Si seen within the leaves of *Festuca* species. This is perhaps unsurprising given the numerous factors, including plant genotype, biotic stresses such as herbivory and abiotic ones such as water availability, known to affect Si levels in plants (Soininen et al., 2013; Quigley and Anderson, 2014).

INTERSPECIFIC DIFFERENCES

Our hypothesis that abrasive species would have higher foliar Si concentration than less abrasive species was not well-supported: *D. cespitosa* has previously been reported to have high leaf Si and to be more abrasive than either of the *Festuca* species, but in this study had lower leaf Si concentration than *F. ovina* and *F. rubra*. Differences in experimental conditions, and hence in plant growth rate, and in plant age, size, and genotype (Soininen et al., 2013) may explain changes in the relative Si concentrations between species, but it is clear that our second hypothesis, namely that more abrasive species had larger, sharper and/or a greater number of spines and phytoliths is supported (**Figure 5**). SEM images revealed that *D. cespitosa* leaves are covered in Si-rich leaf spines which were absent from the leaf surfaces of *F. rubra* (and from *F. ovina* in the absence of additional Si). This strongly suggests that the leaf spines were significantly influencing the abrasiveness of *D. cespitosa* and that phytolith morphology may be more important than leaf Si concentration in determining the abrasiveness of leaves and thus the effectiveness of anti-herbivore defense.

There was a change in morphology and an increase in the number of Si-rich bodies deposited on the leaf surface of *D. cespitosa* and *F. ovina* when plants were provided additional Si; in the case of *D. cespitosa*, phytoliths which had not been present in control leaves were deposited (silica short cells), whereas for *F. ovina*, new, Si-enriched spines were produced, again when spines were not apparent on control leaves. This suggests that these plant species have the ability to deposit new types of Si-based structures to potentially increase their anti-herbivore defenses, whether via abrasion, digestibility effects or both, when Si supply is increased. These changes were most obvious when leaves were also damaged, although interestingly damage in the absence of additional Si did not produce them. In addition to changes in the nature of the spines, the EDX demonstrates that *D. cespitosa* deposited Si only at the tips of spines under control conditions, but under the Si addition treatment, the spines contain Si throughout and the leaf surface is also heavily silicified. A similar pattern was observed in *F. ovina* (**Figure 5**).

Our results support our predictions about the influence of Si supply on the level of Si-based defenses (also see Garbuzov et al., 2011), but damage had less effect on Si-based defenses than we predicted. Although damaged plants were found to have an increase in leaf Si concentration, this was far smaller than the effect of Si supply and there was little effect of damage on spine formation. This may reflect the fact that we used a clipping treatment; simulated damaged may not bring about the same response in spine/phytolith morphology and Si accumulation as herbivory, as reported in previous studies (Massey et al., 2007b). *F. rubra* demonstrated a greater Si uptake to damaged leaves on damaged plants than other species did, which suggests plant species show differences in the way they distribute their Si between different plant parts in response to damage (and potentially other stresses).

The mechanism for this is currently unknown, but there have been reports of between species differences in the ability to load Si into the xylem (Ma and Yamaji, 2006).

CONCLUSION

There were marked differences in the way that even grass species from the same genus deployed the Si they take up in terms of its deposition in structures likely to affect their anti-herbivore defenses. Differences in the localization and the Si-based structures formed has been demonstrated before between plant families (Currie and Perry, 2007), but to our knowledge this is the first time such striking variation has been observed between grass species from the same genus. *F. rubra* had the highest foliar Si content and deposited more Si in damaged leaves than the other two species when plants were damaged under conditions of increased Si supply. However, it is the least abrasive species, presumably because its Si is deposited smoothly and evenly on the leaf surface and not in spines, and any phytoliths produced are few in number and, in contrast to spines, do not protrude substantially above the leaf surface. *D. cespitosa* has a very different strategy: a lower foliar Si content which was less affected by damage and Si addition, but what Si was present was deposited in numerous large spines, particularly at the tip, and under conditions of high Si supply, in a high density of additional structures which are absent under low Si supply. These structures may explain why this species has frequently been shown to be abrasive and unpalatable. Our results suggest that quantifying leaf Si concentration will not give a complete understanding of Si-based anti-herbivore defenses; rather examining how that foliar Si is deposited on the leaf surface will provide a better knowledge of how different plants use their Si and its likely impact on herbivores.

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Silicon, endophytes and secondary metabolites as grass defenses against mammalian herbivores

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Grasses have been considered to primarily employ tolerance in lieu of defense in mitigating damage caused by herbivory. Yet a number of mechanisms have been identified in grasses, which may deter feeding by grazers. These include enhanced silicon uptake, hosting of toxin-producing endophytic fungi and induction of secondary metabolites. While these mechanisms have been individually studied, their synergistic responses to grazing, as well as their effects on grazers, are poorly known. A field experiment was carried out in 5 × 5 m outdoor enclosures to quantify phytochemical changes of either endophyte-infected (E+) or endophyte-free (E−) meadow fescue (*Schedonorus pratensis*) in response to medium intensity (corresponding with densities of ca. 1200 voles/ha for 5 weeks during 3 months) or heavy intensity (ca. 1200 voles/ha for 8 weeks during 3 months) grazing by a mammalian herbivore, the field vole (*Microtus agrestis*). A laboratory experiment was then conducted to evaluate the effects of endophyte infection status and grazing history of the grass diet on vole performance. As predicted, grazing increased foliar silicon content, by up to 13%. Grazing also increased foliar levels of phosphorous and several phenolic compounds, most notably those of the flavonols isorhamnetin-diglycoside and rhamnetin derivative. Silicon concentrations were consistently circa 16% higher in E+ grasses than in E-grasses, at all levels of grazing. Similarly, concentrations of chlorogenic acid derivative were found to be consistently higher in E+ than in E− grasses. Female voles maintained on heavily grazed grasses suffered higher mortality rates in the laboratory than female voles fed ungrazed grass, regardless of endophyte infection status. Our results conclusively demonstrate that, in addition to tolerance, grasses employ multi-tiered, effective defenses against mammalian grazers.

Keywords: defense, endophytes, grasses, grazing, phenolics, secondary metabolites, silicon, voles

INTRODUCTION

Grasses are highly tolerant to grazing by means of their rapid regrowth capacity, basal meristems, underground storage organs and tillering capacity (Dyer et al., 1991; Karban and Baldwin, 2007). Indeed, grasses have been considered to primarily rely on these tolerance traits in lieu of physical or chemical defenses to mitigate damage caused by herbivory. However, a number of mechanisms have been identified in grasses that may deter feeding by grazers, and hence act as defenses (Vicari and Bazely, 1993). The most prominent of these is enhanced silicon (Si) uptake in response to damage (McNaughton and Tarrants, 1983; Massey et al., 2007a).

Following herbivore damage, Si is drawn from the soil and deposited systemically in the cell walls or lumina of new shoots as silica bodies or phytoliths (Ma and Yamaji, 2006; Currie and Perry, 2007). These forms of Si are extremely hard, and thereby increase the abrasiveness of leaf material (Massey and Hartley, 2006), potentially leading to accelerated tooth wear in grazers. High Si levels also inhibit the absorption of nitrogen from digested plant material by mammalian herbivores (Massey and Hartley, 2006).

Grazers have often been observed to avoid plant material with high Si content (Gali-Muhtasib et al., 1992; Cotterill et al., 2007; Reynolds et al., 2012). However, the effects of Si on mammalian herbivore feeding behavior appear in many cases to be moderated or even over-ridden by a range of other factors including species identity (of both grass and herbivore), genotype or growing environment and other aspects of forage quality (Shewmaker et al., 1989; Massey et al., 2009; Soininen et al., 2013a).

Many grass species harbor systemic, vertically transmitted endophytic fungi, which may also increase the resistance of the host to herbivory (Clay, 1988; Cheplick and Clay, 1988; Saikkonen et al., 1998, 2006). Endophytic fungi are often producers of mycotoxins, which either reduce the palatability of the grass or render it toxic to the herbivore (Clay, 1988; Powell and Petroski, 1993; Siegel and Bush, 1996; Saikkonen et al., 1998, 2006). Both the fungal load of grasses and the amount of toxins they produce may increase in response to herbivory, and hence act as induced defenses (Bazely et al., 1997).

While adverse effects of endophyte consumption are well documented for domestic livestock (reviewed by Clay, 1988), results from studies on wild mammalian grazers are scarce and ambiguous at best. Some studies report negative effects, such as decreases in population density (Coley et al., 1995), increased toxicity-induced mortality (Conover, 1998) and suppression of reproduction and growth (Jackson et al., 1996; Durham and Tannenbaum, 1998; Fortier et al., 2000; Conover, 2003), while others have found no effects at all (Barger and Tannenbaum, 1998; Saari et al., 2010). Thus, the effects of endophytic fungi on herbivores may be dose-dependent and may vary between grass, fungi and herbivore species.

Grasses are known to produce an array of secondary metabolites, for example hydroxamic acids (Niemeyer, 1988), condensed tannins (Bernays et al., 1989), cyanogenic glycosides (Jones, 1998) and alkaloids, albeit at levels much lower than dicotyledons (Coughenour, 1985; Vicari and Bazely, 1993). Secondary metabolites found in grasses have been shown to have adverse effects on the performance of rodents when consumed in artificial diets (Lindroth and Batzli, 1984). However, only rarely have these compounds had measurable negative impacts on free-ranging mammalian herbivores at the concentrations in which they naturally occur in grasses. Exceptions again involve livestock: cyanogenic glycosides poisoning cattle (Georgiadis and McNaughton, 1988) and indole alkaloids reducing the palatability of grass for sheep (Simons and Marten, 1971; Marten et al., 1973).

Most studies have failed to identify significant effects of grazing on levels of defensive secondary metabolites in grasses (Lindroth and Batzli, 1986; Klemola et al., 2000). Nonetheless, graminivorous herbivores are highly selective feeders both within and between plant species (Freeland, 1974; Hjältén et al., 1996). As this behavior is to a great extent governed also by the secondary chemistry of their food plants (Jung and Batzli, 1981; Bergeron and Joudoin, 1989; Massey et al., 2007b), variation in grass secondary metabolite concentrations holds the potential to influence grazing mammals and their population demography (Freeland and Janzen, 1974).

Although these three potential defensive mechanisms of grasses – Si induction, endophytes, and secondary metabolites – have been individually studied, their synergistic responses to grazing and interactive effects on herbivores are poorly known. The aim of this study was to simultaneously quantify how levels of Si, other nutrients and secondary metabolite content interact in grasses in response to mammalian grazing and endophyte infection status. Furthermore, the aim was to evaluate the synergistic effect of these potential defense mechanisms on the physiological performance of a graminivorous herbivore. Although all of the grass defenses focussed on in this study are mechanisms through which herbivore populations may potentially be affected, the evidence for impacts of mechanisms in natural populations is missing.

Meadow fescue (*Scherodonus pratensis* ex. *Lolium pratense* and *Festuca pratensis*), which is commonly infected by the endophytic fungus *Epichloë uncinata* (Saari et al., 2009) was used as a model grass species. As an indicator of secondary metabolite production, we analyzed levels of phenolics, a widespread group of important defensive compounds (Rhoades, 1979) known to have adverse effects on graminivorous herbivores (Lindroth and Batzli, 1984). A common European grassland rodent, the field vole *Microtus agrestis* served as our model grazing herbivore.

The following specific hypotheses were tested: (1) vole grazing elevates levels of both Si and phenolics in grasses, (2) the magnitude of response of Si and phenolics to grazing differs between endophyte-infected and non-infected grasses, and (3) consumption of heavily grazed grass, as compared to consumption of less heavily grazed grasses, has negative impacts on vole physiological condition, and more so if the grass is endophyte-infected.

MATERIALS AND METHODS

STUDY SPECIES

Meadow fescue is one of the most important native forage grasses in Nordic countries. It is also common outside of agronomic use in meadows, roadsides, and wastelands (Hämet-Ahti et al., 1998). Meadow fescue cultivars are often colonized by the systemic endophyte *E. uncinata*, which produces loline alkaloids, which appear to be non-toxic to large mammal herbivores (Clay and Schardl, 2002) but can be noxious to invertebrates and small vertebrates (Conover, 2003; Saikkonen et al., 2006; Huitu et al., 2008). A common meadow fescue cultivar “Kasper,” registered and commercialized for use in Nordic countries in 1989 (Saari et al., 2009) was used for purposes of this study.

The field vole is a common and widespread small rodent species in Europe, including Fennoscandia (Myllymäki, 1977). It exhibits high-amplitude 3–4 year population cycles which are synchronous across large geographic areas (Sundell et al., 2004; Korpela et al., 2013). Field voles preferentially inhabit grassland habitats (Hansson, 1971; Myllymäki, 1977). Their diet consists predominately of grasses and forbs (Hansson, 1971).

EXPERIMENTAL DESIGN AND PROTOCOL

The study was conducted in fenced enclosures in Jokioinen, South–West Finland (60°49′ N, 23° 30′ E) during summer 2011. The enclosures (20 in total in 5 × 4 configuration, each 25 m × 39 m; **Figure 1**) were established in May 2006 in an old agricultural field by tilling, fertilizing with cow manure (30 000 kg/ha) and sowing as monocultures either endophyte-free seed lots (E−; 0% endophyte frequency) or endophyte-infected seed lots (E+; 79% infection frequency) of the meadow fescue cultivar “Kasper” at a rate of 20 kg ha^{-1} (Saikkonen et al., 2013). In June 2007, plots were fertilized again with a commercial fertilizer [16:9:22 (N:P:K) with micronutrients, Kemira, product number: 0647334]. E+ and E− treatments were randomly assigned to 10 enclosure pairs. Seed lots were obtained from seed production farms via the Finnish Food Safety Authority (EVIRA), Seed Certification Unit, Loimaa, Finland. Four years after establishment, the cover percentages of meadow fescue had decreased from 100 to 75% and 98% in E– and E+ plots, respectively, due to weed invasion (Saikkonen et al., 2013).

Each enclosure was constructed of a sheet metal fence (embedded 60 cm below ground while 60 cm remained above ground) in

FIGURE 1 | Aerial photo of experimental enclosure complex. The denotation E+ indicates those 25 × 39 m enclosures which were vegetated by endophyte-infected meadow fescue, and E− those with endophyte-free meadow fescue. The denotation G+ in the smaller plots pictured in the inset indicate those plots in which field voles grazed through the summer, and G− those, which were not grazed. Photo courtesy of the National Land Survey of Finland.

order to retain the experimental voles and exclude voles of natural populations and small mammal predators.

Every second enclosure pair of the 10 available pairs was selected for the experiment (**Figure 1**). Four 5m × 5 m sheet metal plots arranged in 2 × 2 squares were constructed (**Figure 1**) in one randomly assigned corner (corners bordering the outside of the enclosure complex were excluded to reduce predator attraction) of each of the 10 enclosures (5 × E+ and 5 × E−). Two plots per enclosure were randomly designated as vole grazing plots (G+) and two as non-grazed control plots (G−).

In total, the experiment consisted of 10 replicates of each of the following factorial treatments: vole grazing in endophyte-infected plots (G+E+), vole grazing in endophyte-free plots (G+E−), no grazing in endophyte-infected plots (G−E+), and no grazing in endophyte-free plots (G−E−). The grazing treatment was begun in late May 2011 by introducing two adult field voles, trapped in the vicinity of the enclosures and in Suonenjoki, Eastern Finland, into each G+ plot. Prior to introduction, all voles were housed in standard laboratory rodent cages (43cm × 26cm × 15 cm; Tecniplast, Italy) with ad libitum water, feed pellets (Altromin 1314F; Altromin Spezialfutter GmbH and Co. Germany), and turnip.

Two levels of grazing intensity we applied to ensure adequate responses of grasses to the grazing treatment. The medium grazing intensity treatment involved two voles grazing in each plot continuously for 2 weeks, and thereafter four voles grazing for 3 weeks, either directly after the first 2 weeks, or after a 3 week period of no grazing. The heavy grazing intensity treatment involved two voles grazing in each plot continuously for 2 weeks, and thereafter four voles grazing first for 3 weeks, followed by 3 weeks of no grazing and finally another 3 weeks of grazing by four voles.

In summary, the medium grazing intensity plots received 14 days of grazing at a vole density corresponding to 800 voles / ha (2 voles × 0.0025 ha = 800 voles/ha) plus 21 days of grazing at a vole density corresponding with 1600 voles/ha (4 voles × 0.0025 ha = 1600 voles/ha). The high intensity grazing treatments received 14 days of grazing at 800 voles / ha plus 42 days of grazing at 1600 voles/ha. The collective grazing intensity over the experiment approximated 1200 voles/ha for 5 weeks during 3 months and ca. 1200 voles / ha for 8 weeks during 3 months for the medium and heavy grazing intensity treatments, respectively. The level of grazing on individual grasses was not measured, but visual inspection indicated that while heavy grazing did have a substantial effect on vegetation, grasses were at no point during the experiment entirely deplete within the plots.

Live trapping was conducted in mid-June in all enclosures to measure the body mass of voles in the G+ enclosures and to verify that no voles had entered the G− enclosures. As expected, no voles were encountered in the G− enclosures. However, only circa 25% of the originally introduced voles were trapped in the G+ enclosures over three nights. This strongly indicated predation within the experimental enclosures, and a cat was occasionally seen around the enclosure complex.

Therefore, the experimental grazing protocol was altered from its original form. In mid-June, all voles were removed from the plots. Four field voles were then introduced into half of the G+ plots for a period of 3 weeks. Thereafter, the voles were trapped and translocated to the remaining plots. This was again repeated after 3 weeks, with four voles returned to the plots that were first grazed. During this 9 week period, only seven voles disappeared, and those that did were replaced upon translocation. All voles were live trapped out of the plots at the end of August.

SAMPLE COLLECTION AND PHYTOCHEMICAL ANALYSES

To evaluate the effects of grazing on phytochemistry, meadow fescue samples were collected in each of the plots three times during the summer: immediately prior to introduction of voles, once at the beginning of August and once at the beginning of September (1 week after voles had been removed). Each time, one tiller was removed at the base from three randomly selected meadow fescue plants per plot and pooled into one sample. During the first sampling occasion, samples from the two G+ and two G− plots per enclosure were also pooled.

The Si, phosphorous (P), nitrogen (N), and carbon (C) contents of the samples were analyzed for each of the three sampling occasions. For these analyses, grass samples were washed under water, oven dried at 80°C and ground into a fine, non-fibrous powder. Three hundred milligram of this powder was pressed in a hydraulic press into a 3 mm thick, 13 mm diameter pellet with a pressure of 13 bar. Si and P content (expressed as percentage dry weight) was analyzed from the pellets with a portable X-ray fluorescence spectrometer (Niton XL3t900 GOLDD Analyzer; Thermo Scientific, Winchester, UK), as described by Reidinger et al. (2012).

As this technique is non-destructible, the same pellets were used for the analysis of N and C. This was done by ISO 13878 and ISO 10694 based methods, respectively, using a LECO/CHN analyser (Leco Co., USA).

Phenolic compounds were analyzed only from samples collected on the last occasion, after the grazing treatment. These samples were air dried at room temperature for 1 week prior to analyses. All soluble non-tannin phenolic compounds were extracted and HPLC-analyzed from the grass leaf samples according to the protocol described in Nybakken et al. (2012).

VOLE LABORATORY EXPERIMENT

At the beginning of September, a laboratory experiment was carried out to evaluate how earlier grazing and endophyte infection status affected vole physiological condition and performance. 96 wild field voles, captured near the towns of Suonenjoki, central Finland (62° N, 27° E) and Jokioinen, were housed in same sex pairs in a decommissioned greenhouse, in standard laboratory cages containing peat and straw as bedding and nest material. The voles were assigned to one of four food treatment groups ($n = 24$ voles per treatment group), which received *ad libitum* grass collected daily from the experimental plots (treatments: E+G+, E+G−, E−G+, and E−G−). For this segment of the study, all grass collected from grazed enclosures was pooled and not distinguished between heavy or medium grazing. Voles also received pieces of potato for extra hydration.

Experimental feeding began on September 10th and continued until the conclusion of the experiment on September 26th. Throughout this period, voles were checked daily for survival, and each animal was weighed using a spring balance (Pesola AG, Switzerland; accuracy ± 1 g) every 3–4 days (six measurement occasions in total). In the beginning and at the end of the feeding period, a blood sample was collected from the retro-orbital sinus of each vole using a capillary tube. Tubes were then centrifuged at 12000 g for five minutes, and haematocrit expressed as the percentage of packed red blood cells in total volume. The feeding experiment was terminated and all voles euthanized due to an increased rate of mortality in the end of the second experimental week, for reasons that remain unidentified.

The experiment was carried out under permit from the Animal Ethics Council of the State Provincial Office of Southern Finland.

STATISTICAL ANALYSES

Variation in grass Si, P, N, and C content was analyzed with linear mixed models using the maximum likelihood method, where each of the elements was in turn the response variable. Grazing intensity (heavy, medium or control), endophyte infection status (E+ or E−), time (June, August, September) and all interactions were initially entered in the models as class explanatory variables. Time was designated a repeated factor, with plot as the subject. In all cases, AIC-based model selection (Burnham and Anderson, 2000) indicated unstructured as the most parsimonious covariance type. Enclosure was entered as a random factor.

Variation in phenolics concentrations at the end of summer was also analyzed with separate linear mixed models for each compound using the maximum likelihood method. Initially, all peak intensity values for the different compounds were $\log_e$ -transformed. Grazing intensity, endophyte infection status and their interaction were entered as fixed explanatory variables, while enclosure and plot (nested within enclosure) were designated random factors.

The overall response of meadow fescue to the treatments in terms of their phenolic content was also analyzed with multivariate analysis of variance using all 16 identified phenolic compounds as response variables, and grazing intensity, endophyte infection status and their interaction as explanatory variables.

Changes in the body mass and haematocrit value of voles that survived to the end of the laboratory experiment were analyzed with linear mixed models, with the percentage of change relative to the beginning of the experiment as the response variable. Grazing history (grazed or not) and endophyte infection status of the grass they were fed, vole sex and body mass at the beginning of the experiment, together with all possible three-way interactions, were entered as fixed explanatory variables. Identity of the cage in which the voles were housed was entered as a random factor. Full models were reduced by removing explanatory terms on the basis of AIC. Model fit was assessed by visual observation of the final model residuals.

Vole survival during the laboratory experiment was analyzed with generalized linear mixed models, using vole survival as a binary response variable with a logit link. Grazing history and endophyte infection status of the grass, vole sex, and all interactions, were entered as fixed explanatory variables and vole cage as a random factor.

Full models were reduced in all analyses by removing explanatory terms on the basis of AIC – terms were removed if this reduced the AIC value by >2 units (Burnham and Anderson, 2000). Model fit was assessed in the linear mixed models by visual inspection of the final model residuals, and by the ratio of the generalized chi-square statistic and its degrees of freedom in the generalized linear mixed model. All analyses were carried out with SAS statistical software v9.3 (SAS® Institute Inc., Cary, NC, USA).

RESULTS

Grazing elevated the Si content of grasses ($F_{2,24.4} = 3.50$, $p = 0.046$). Highest levels were recorded in the heavy grazing treatment (mean ± SE. Si content as percentage dry weight = 1.56 ± 0.07%), followed by medium grazing (1.54 ± 0.08%), while control grasses had the lowest values (1.36 ± 0.05%; **Figure 2**). Si levels were consistently higher in E+ grasses than in E− grasses, with no significant interactive effect of grazing (E+: mean ± SE. Si content as percentage dry weight = 1.60 ± 0.06%; E− = 1.38 ± 0.06%; **Figure 2**).

Likewise, grazing elevated the P content of grasses ($F_{2,28.4} = 3.78$, $p = 0.035$). Highest levels were recorded in the medium grazing treatment (mean ± SE. P content as percentage dry weight = 0.42 ± 0.01%), followed by heavy grazing (0.42 ± 0.01%), while control grasses had the lowest values (0.40 ± 0.01%; $t^{\text{control vs. heavy}}{}_{24.8} = -2.03$, $p = 0.054$; $t^{\text{control vs. medium}}{}_{31.2} = -2.43$, $p = 0.021$; $t^{\text{heavy vs. medium}}{}_{30.5} = -0.35$, $p = 0.73$). The level of P declined in E+ grasses over the summer, whereas that in E− grasses remained stable ($F_{2,27.6} = 3.79$,

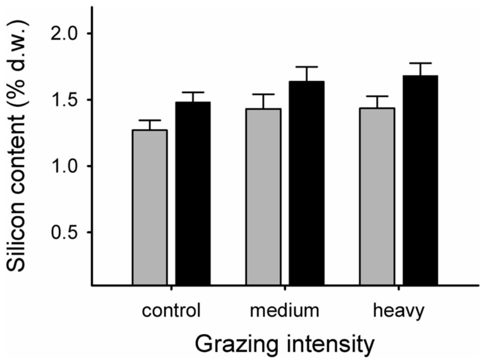

FIGURE 2 | Silicon content, expressed as percent dry weight in endophyte-free (E−; gray bars) and endophyte-infected (E+; black bars) grasses from control, medium grazing and heavy grazing intensity enclosures at end of summer. Differences between grazing intensity treatments: $t^{\text{control vs. heavy}}{}_{18.1} = -2.38$, $p = 0.028$; $t^{\text{control vs. medium}}{}_{28.5} = -1.83$, $p = 0.079$; $t^{\text{heavy vs. medium}}{}_{30.3} = 0.24$, $p = 0.81$. Differences in Si content between endophyte-infected and non-infected grasses: $F^{\text{endophyte}}{}_{1,9.33} = 6.97$, $p = 0.026$).

$p = 0.035$). No differences were found in concentrations of N, C, or in C/N-ratio relative to grazing intensity, endophyte infection status, time or their interactions (all p-values > 0.08).

A two-way multivariate analysis of variance revealed a significant interaction between grazing intensity and endophyte infection status on the phenolics profile of the grasses (Wilks' Lambda $= 0.12$, $F_{32,38} = 2.27$, $p = 0.008$). One-way multivariate tests with either E+ or E− samples did not clearly indicate which of the main effects had a stronger effect on grass phytochemistry. The varying degrees of grazing intensity tended to influence the phenolics profile of endophyte-free samples (Wilks' Lambda $= 0.001$, $F_{32,4} = 4.58$, $p = 0.074$), but these differences were less pronounced in endophyte-infected samples (Wilks' Lambda $= 0.02$, $F_{32,4} = 2.40$, $p = 0.21$).

Five of the 16 analyzed phenolic compounds exhibited an elevated response to grazing – chlorogenic acid, isorhamnetin-diglycoside, myricetin-glycoside, isorhamnetin 3-glucoside, and rhamnetin derivative (**Table 1**). Concentrations of chlorogenic acid derivative were higher in E+ than in E− grasses. Two compounds were affected by the interaction between grazing intensity and endophyte status. Levels of quercitrin, in particular, were positively associated with grazing intensity, but only in E+ grasses (**Table 1**).

The body mass of voles maintained in the laboratory on endophyte-infected and endophyte-free grasses responded differently to the grazing history of their diet. Voles feeding on endophyte-free grass that had been grazed throughout the summer lost relatively more mass than voles in the other three groups (mean ± SE. mass change during experiment as percentage: E−G− $= +3.90 \pm 3.12\%$, E−G+ $= -8.92 \pm 3.08\%$, E+G− $= +0.74 \pm 2.88\%$, E+G+ $= +0.62 \pm 3.18\%$). Feeding on previously grazed grass also had a significant negative main effect on vole body mass change (mean ± SE. mass change: G− $= +2.32 \pm 2.15\%$, G+ $= -4.15 \pm 2.18\%$). Females lost more body mass than males, irrespective of dietary group (mean ± SE. mass change: males $= +3.21 \pm 2.26$, females $= -5.04 \pm 2.06\%$; **Figure 3**).

Females that had fed on endophyte-infected grass had elevated haematocrit levels compared to those fed on endophyte-free grasses (mean ± SE.: E+ $= 51.3 \pm 0.8$, E− $= 48.9 \pm 0.9$; $F_{1,19.7} = 4.55$, $p = 0.046$). Conversely, females maintained on heavily grazed grasses suffered ca. 45% higher mortality rates than females fed ungrazed grass, regardless of endophyte infection status; these differences did not manifest in males (**Figure 4**).

DISCUSSION

In support of our hypotheses, vole grazing elevated levels of both Si and phenolics in grasses. Endophyte infection alone also elevated grass Si content, to an extent comparable to that of grazing. However, changes in the levels of Si in response to grazing did not differ between E+ and E− grasses. Grazing and endophyte infection status did interact in altering the phenolic compound profile of grasses. Also in support of our hypothesis, consumption of heavily grazed grass had negative impacts on vole physiological condition, but these effects were conditional on endophyte infection status. Although endophyte infection elevates Si content as much as grazing alone does, our results overall suggest that grazing has a more prominent effect on grass defenses than endophyte infection status.

GRASS RESPONSES TO GRAZING

As predicted, grazing-induced the uptake of Si from the soil into the grass. This response has been documented several times in association with various grazer taxa (McNaughton and Tarrants, 1983; Massey and Hartley, 2006; Massey et al., 2007a), and the biochemical processes underlying it are well known (Ma and Yamaji, 2006; Currie and Perry, 2007). Si accumulation has been identified as the most plausible, and potent defense mechanism of grasses against graminivorous voles (Massey and Hartley, 2006). It has even been suggested as a candidate factor for generating multi-annual population cycles in rodents, as its induction in response to grazing may occur with a delay relative to rodent density, and the induced levels of Si persist in the grasses for sufficiently long (Massey et al., 2008; Reynolds et al., 2012).

Although the accumulation of Si has in many study systems received support as a defense mechanism against grazers, many others have failed to do so. For example, Shewmaker et al. (1989) could not demonstrate any effects of Si content on the grazing preference of sheep, which appear to be less responsive to variations in grass Si content than small rodents (Massey et al., 2009). Densities of African grazing ungulates have been shown to be either negatively (McNaughton and Tarrants, 1983) or positively (Georgiadis and McNaughton, 1990) associated with the Si content of their food plants. This contingency in responses is likely to reflect differences in grazing levels and hence in the magnitude of Si induction, given the known impacts of both frequency and intensity of damage on the Si response (Massey et al., 2007a; Reynolds et al., 2012), as well as variations in the range of plant, herbivore and environmental factors influencing plant-herbivore interactions (e.g., Soininen et al., 2013a).

In subarctic Fennoscandia, where vole densities are 2–3 orders of magnitude lower than simulated in this experiment, grass Si

Table 1 | Mean peak intensity values of the phenolic compounds analyzed, grouped by grazing intensity, and endophyte status of the grasses.

Phenolic compound	Grazing intensity						Significant effect	Direction
	Control		Medium		Heavy			
	E−	E+	E−	E+	E−	E+		
Gentisic acid	0.12	0.10	0.08	0.09	0.09	0.10	n.s.	
Neochlorogenic acid	0.80	0.91	0.97	0.88	0.65	0.87	n.s.	
Chlorogenic acid	11.35	10.80	12.10	13.01	11.12	12.11	grazing	C < M ($p = 0.057$)
Chlorogenic acid derivative	0.12	0.17	0.13	0.16	0.11	0.15	endophyte	E− < E+, $p = 0.051$
Quercetin-diglycoside	1.36	1.12	1.37	1.42	1.23	1.34	n.s.	
Isorhamnetin-diglycoside	0.70	0.56	0.77	0.87	0.74	0.88	grazing	C < H and M
Myricetin-glycoside	0.01	0.01	0.01	0.02	0.03	0.04	grazing	H > C and M
Isorhamnetin-glycoside	0.02	0.03	0.03	0.04	0.06	0.05	n.s.	
Quercetin-glucoside	1.29	1.59	1.66	1.85	1.36	2.11	n.s.	
Kaempferol-glycoside	0.02	0.01	0.02	0.04	0.02	0.03	n.s.	
Quercitrin	0.03	0.01	0.03	0.05	0.03	0.06	interaction	C E− > C E+; H E+ > E−; C E+ < M E+ and H E+
Kaempferol 3-glucoside	0.07	0.10	0.11	0.11	0.09	0.11	interaction	C E− < M E−; C E− < M E+ ($p = 0.054$)
Isorhamnetin 3-glucoside	0.27	0.35	0.44	0.51	0.36	0.62	grazing	C < M and H
Rhamnetin derivative 1	0.20	0.20	0.21	0.23	0.20	0.23	n.s.	
Quercetin derivative	0.01	0.01	0.02	0.04	0.02	0.03	n.s.	
Rhamnetin derivative 2	0.11	0.12	0.13	0.15	0.12	0.14	grazing	C < M ($p = 0.008$) and H ($p = 0.059$)

Significant effect denotes which of these terms was significant at a risk level of $\alpha = 0.05$ *(borderline values indicated in column Direction). Direction denotes pairwise differences between levels of the significant effect and their direction; C, M, and H denote control, medium and heavy grazing intensities, E+ and E− indicate endophyte-infected and non-infected grasses, respectively.*

levels appear to be influenced more by grass species, their genotype or their growing environment than by the mostly low grazing pressure (Soininen et al., 2013a). Indeed, the majority of experimental studies in which Si has become induced in response to rodent grazing have been conducted in laboratory or greenhouse conditions (Massey and Hartley, 2006; Massey et al., 2007a; Reynolds et al., 2012, but see Massey et al., 2008), or as is the case here, in small outdoor enclosures. In these experimental conditions it is relatively easy to impose high damage levels in terms of biomass removed (Reynolds et al., 2012) or high vole densities (this study). It seems likely that both vole densities and the frequency and intensity of plant damage are likely to be lower in natural systems, which are also subject to a greater range of environmental variability (including climatic factors, soil type, plant genotypes, and growth stage), making the detection of Si induction in the field at the landscape scale more challenging.

While no differences were found in concentrations of N, C, or in C/N-ratio relative to grazing intensity or endophyte infection status, grazing did elevate the P content of grasses. Levels of phosphorous have been found to increase in response to grazing in grasses, but this primarily occurs to facilitate shoot regrowth after defoliation, rather than to act as an induced defense against grazers (Chapin and Slack, 1979; Donaldson et al., 1984; McNaughton and Chapin, 1985). It is evident that the primary chemistry of the grasses did not exhibit pronounced changes in response to defoliation by grazing.

Grazing increased the content of five of the 16 analyzed phenolic compounds – chlorogenic acid and five flavonols, isorhamnetin-diglycoside, myricetin-glycoside, isorhamnetin 3-glucoside, and rhamnetin derivative (**Table 1**). Many phenolics are inducible defensive compounds, which either inhibit digestion by herbivores, are deterrent or even directly toxic (Lindroth and Batzli, 1984; Karban and Baldwin, 2007). Their concentrations in grasses are generally low relative to other plant taxa (Bernays et al., 1989) and their collective levels appear not to respond greatly to grazing, at least by rodents (Lindroth and Batzli, 1986). However, in response to herbivory, the relative concentrations of different individual phenolic compounds may change dramatically without changing total concentrations, and it has been demonstrated that individual compounds can impact adversely on voles (Lindroth and Batzli, 1984).

A clear caveat of our study on grass phytochemical responses to grazing is the fact that concentrations of all possible defensive compounds, such as cyanogenic glycosides, hydroxamic acids, or alkaloids were not comprehensively analyzed. A number of these have been found to respond to grazing and to have adverse effects

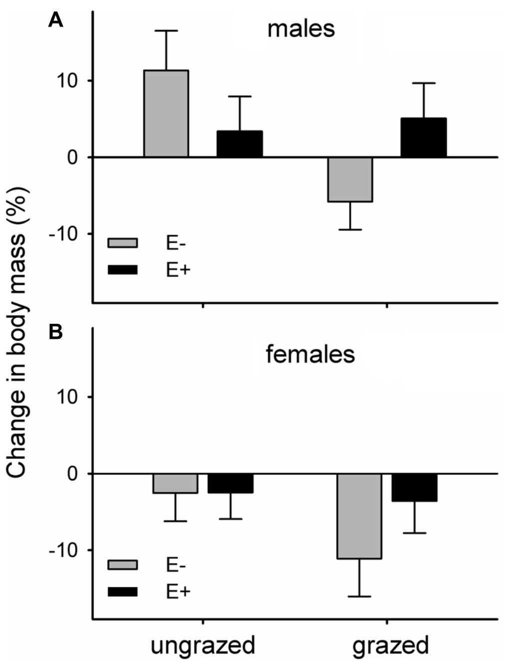

FIGURE 3 | Changes in body mass of voles maintained in the laboratory on previously grazed or ungrazed endophyte-infected (E+) or endophyte-free (E−) grass; (A) males, (B) females. Differences of changes in body mass of voles relative to the treatments and vole sex: $F^{\text{endophyte} \times \text{grazing}}_{1,40.9} = 4.14$, $p = 0.049$; $F^{\text{grazing}}_{1,38.8} = 4.38$, $p = 0.043$; $F^{\text{sex}}_{1,40.3} = 7.20$, $p = 0.01$.

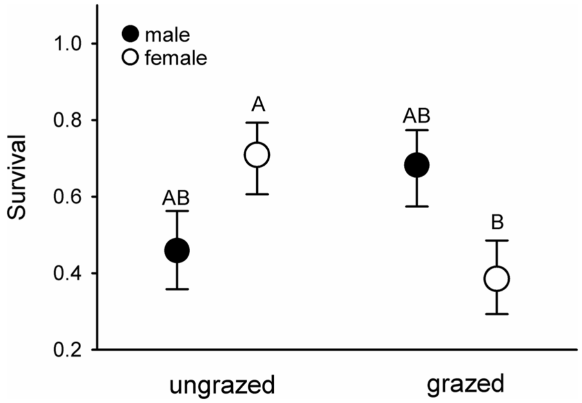

FIGURE 4 | Survival of male (filled symbols) and female (open symbols) voles maintained in the laboratory on previously ungrazed or grazed grass. Letters above the symbols denote significant differences between the groups at $\alpha < 0.05$. $F^{\text{sex} \times \text{grazing}}_{1,45.41} = 6.83$, $p = 0.013$.

on herbivores (reviewed by Vicari and Bazely, 1993). The nature of these associations in our study system remains to be investigated in future research.

EFFECTS OF ENDOPHYTE INFECTION ON GRASS PHYTOCHEMISTRY

Si levels were found to be to be slightly higher in E+ grasses than in E− grasses. Si has been shown to be positively associated with drought resistance in grasses, as it forms physical structures on leaves which reduce transpiration through stomata (Lux et al., 2002). Si is also known for its capacity to inhibit the growth of pathogenic fungi (Fauteux et al., 2005) and although often described as having mutualistic rather than antagonistic interactions with its host plants (Saikkonen et al., 2006), it is plausible that endophytic fungi are in some cases identified by their hosts as harmful pathogens (Saikkonen et al., 1998, 2004). An alternative explanation for the elevated Si levels observed in E+ grasses in this study may therefore be an evolutionarily conserved defensive response against the intrusion of a foreign fungal organism, even potentially mutualistic ones.

Similarly, chlorogenic acid derivative levels were higher in E+ than in E− grasses. Chlorogenic acid has been shown to act as a chemical defense against invertebrate herbivores (Bernays et al., 2000; Ikonen et al., 2001), though very little is known of how, or if, chlorogenic acid acts as a defense against fungal infections in plants, as Si does.

INTERACTIVE EFFECTS OF GRAZING AND ENDOPHYTE INFECTION ON GRASS PHYTOCHEMISTRY

Two phenolic compounds were affected by the interaction between grazing intensity and endophyte status. Levels of quercitrin (also known as quercetin 3-rhamnoside), in particular, were positively associated with grazing intensity, but only in E+ grasses (**Table 1**). Quercitrin consumption has been shown to reduce the growth of weanling voles, and most profoundly so when dietary protein levels are low (Lindroth and Batzli, 1984). The effects of quercitrin on voles appear to be toxic, as dietary protein levels have been shown not to affect protein digestibility – rather, low dietary protein levels appear to reduce the capacity of voles to detoxify quercitrin (Lindroth and Batzli, 1984).

The grazing-induced elevation of Si may have more far-reaching effects than the up-regulation of the physical defense of plants. For example, Si appears also to have a functional role in the systemic response of plants to pathogens, through the activation of general defensive pathways, e.g., involving salicylic, or jasmonic acids (Fawe et al., 1998; Rodrigues et al., 2004; Fauteux et al., 2005). Elevated Si levels have also been shown to increase the accumulation of lignin, phenolic compounds, chitinases, and peroxidases (Fawe et al., 1998). As such, Si is likely to be integral in the regulation of terpene or phenolic –based defenses in stressed plants (Fauteux et al., 2005).

A caveat of our study is that we were unable to determine the effects of vole grazing on either the amount of endophyte hyphae per grass leaf or the concentrations of mycotoxin alkaloids that the fungus produces. Bazely et al. (1997) demonstrated that intensive sheep grazing increased hyphae loads and/or alkaloid production. A similar mechanism may occur with vole grazing, though this too remains a topic for further investigation.

EFFECTS OF DIET QUALITY VARIATION ON VOLE CONDITION

Voles that consumed previously grazed grass lost body mass in the laboratory. Massey and Hartley (2006) established that an elevated concentration of Si in the diet is detrimental to graminivorous voles, in that it reduces their capacity to absorb nitrogen from their diet, leading to reduced growth rates in juveniles and adult females, and our study confirms this. Conversely, there are far fewer cases in which secondary metabolites have been shown to have similar negative effects on graminivorous mammals (see Simons and Marten, 1971; Marten et al., 1973; Georgiadis and McNaughton, 1988), let alone voles. Although grasses produce a wide array of secondary metabolites (reviewed by Vicari and Bazely, 1993), their effects on rodents appear to manifest only when animals are fed on experimental diets in laboratory conditions (e.g., Lindroth and Batzli, 1984). In light of this, it seems more parsimonious to attribute the observed body mass loss of voles to elevated Si content in the grasses than phenolics content. However, our experimental design does not enable us to firmly discriminate between these factors.

Females, in particular, suffered mass loss while maintained in the laboratory, regardless of treatment. The voles were offered only one species of grass as diet. Consumption of such a monotonous diet is highly unlikely for voles in natural habitats (Myllymäki, 1977; Soininen et al., 2013b). The fact that females suffered greater mass loss than males may be due to intersexual differences in dietary requirements related to, e.g., reproduction (Barboza and Bowyer, 2000; of note, none of the females in our experiment were gravid). Unfortunately, virtually no information exists on sex differences in dietary intake in rodents.

Females that had fed on endophyte-infected grass had elevated haematocrit levels compared to those fed on endophyte-free grasses. Haematocrit measures the relative volume of red blood cells in whole blood, and its values may increase due to either proliferation of red blood cells, or, more commonly, due to a reduction in blood plasma content due to dehydration (Fair et al., 2007). A dehydration-induced reduction in haematocrit often occurs in association with prolonged fasting in animals (Vleck et al., 2000). It is likely that the ultimate factors which lead to female mass loss and high death rate also contributed proximately to their haematocrit levels. Thereby, it is plausible that females were more sensitive either to the increased Si content of the E+ grasses, or to the endophyte itself, than were male voles.

Highest levels of mortality were recorded for females maintained on grazed grasses. Conversely, endophyte infection status did not affect mortality rates. Although endophyte infection status appeared to be associated with female food avoidance in the laboratory, as judged by hematocrit levels, these effects were not pronounced enough to impact survival. Collectively, our results indicate that grazing-induced changes in the quality of diet, i.e., increased Si and phenolics content, outweigh the potential negative effects of endophyte infection on the physiological condition of voles (see Lindroth and Batzli, 1984; Massey and Hartley, 2006). Furthermore, we demonstrate here that heavy and prolonged grazing may reduce the quality of graminivorous vole diet to the extent that it has negative effects on vole survival. By extension, as the most profound negative impacts were observed in females, these effects hold the potential to carry over to population growth. However, we acknowledge that the artificial nature of our laboratory may have generated spurious associations between food quality and vole performance. More detailed experimental research is therefore still called for to elucidate these causal relationships in entirely natural surroundings.

CONCLUSION

Our results render support to the hypothesis that food quality may indeed have a limiting effect on vole population growth, in cases when grazing is severe and long enough (see Reynolds et al., 2012). We conclusively demonstrate here that grasses are capable of employing multi-tiered, effective defenses against mammalian grazers. We do not imply that these findings are universally applicable to all grazing ecosystems – rather, we highlight the need for more detailed investigations on the entire metabolome and the relative effects of defense mechanisms thus far identified in grasses to elucidate the role of food quality as a determinant of herbivore population dynamics.

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Silica uptake by *Spartina*—evidence of multiple modes of accumulation from salt marshes around the world

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Silicon (Si) plays a critical role in plant functional ecology, protecting plants from multiple environmental stressors. While all terrestrial plants contain some Si, wetland grasses are frequently found to have the highest concentrations, although the mechanisms driving Si accumulation in wetland grasses remain in large part uncertain. For example, active Si accumulation is often assumed to be responsible for elevated Si concentrations found in wetland grasses. However, life stage and differences in Si availability in the surrounding environment also appear to be important variables controlling the Si concentrations of wetland grasses. Here we used original data from five North American salt marshes, as well as all known published literature values, to examine the primary drivers of Si accumulation in *Spartina*, a genus of prolific salt marsh grasses found worldwide. We found evidence of multiple modes of Si accumulation in *Spartina*, with passive accumulation observed in non-degraded marshes where *Spartina* was native, while rejective accumulation was found in regions where *Spartina* was invasive. Evidence of active accumulation was found in only one marsh where *Spartina* was native, but was also subjected to nutrient over-enrichment. We developed a conceptual model which hypothesizes that the mode of Si uptake by *Spartina* is dependent on local environmental factors and genetic origin, supporting the idea that plant species should be placed along a spectrum of Si accumulation. We hypothesize that *Spartina* exhibits previously unrecognized phenotypic plasticity with regard to Si accumulation, allowing these plants to respond to changes in marsh condition. These results provide new insight regarding how salt marsh ecosystems regulate Si exchange at the land-sea interface.

Keywords: *Spartina*, salt marsh, silica, accumulation, grasses, wetland, ecosystem service

INTRODUCTION

The role of nitrogen (N) and phosphorus (P) in plant productivity is well recognized by plant ecologists. Less well constrained is the importance of silicon (Si) in plant growth, maintenance, and fitness (Cooke and Leishman, 2011). In terrestrial ecosystems, Si is classified as a "quasi-essential" nutrient (Epstein, 2009) because it protects plants from a variety of stressors, including desiccation, heavy metal toxicity, and predation (Epstein, 1994; Hodson and Evans, 1995; Liang et al., 2007). Through their roots, plants take up dissolved silica (DSi) (SiO_2) from the porewater, or soil solution, and transport it through the transpiration stream via the xylem. As water leaves the plant, Si becomes concentrated at transpiration termini and is deposited as biogenic Si (BSi). As such, plants often demonstrate increasing BSi concentrations with age or growth (Jones and Handreck, 1967; Epstein, 1994), due to continual precipitation of BSi at transpiration termini and the inability of plants to translocate Si once precipitated (Raven, 1983; Epstein, 1994). Although the majority of Si in plants is located at transpiration termini (e.g., leaves), Si is also found throughout other portions of plants, such as the roots, rhizomes, and stems (Struyf et al., 2005b; Querné et al., 2012; Carey and Fulweiler, 2013). The concentration of Si in plants is often higher than many macronutrients such as N and potassium (K). Si concentrations typically range from 0.1 to 10% by weight (by wt.) (Ma et al., 2001), the largest range of any element found in plants (Epstein, 1994).

The accumulation of Si in plants occurs via three possible modes of uptake: (1) active accumulation, where plants take up more Si than they would through water uptake alone, (2) passive accumulation, where plants have similar Si and water uptake rates, and (3) rejective or excluder accumulation, where Si uptake is slower than water uptake (Raven, 1983; Takahashi et al., 1990; Ma et al., 2001). These three possible modes of Si accumulation can be determined in several ways (Jones and Handreck, 1967; Takahashi et al., 1990; Ma et al., 2001). First, Si accumulation can be defined by the concentrations of Si in the aboveground plant tissue alone, where active accumulators typically have a dry weight of SiO_2 $>1\%$ by wt., passive accumulators between 0.5 and 1% by wt., and excluders $<0.5\%$ by wt. (Ma et al., 2001; Street-Perrott and Barker, 2008; Hou et al., 2010; Carey and Fulweiler, 2012). The ratio of Si to calcium (Ca) is another means of determining the mode of Si accumulation, with Si:Ca ratios >1 indicating active accumulation, 0.5–1.0 indicating passive accumulation, and <0.5 indicating Si exclusion (Takahashi et al., 1990; Ma et al., 2001; Ma and Takahashi, 2002). Finally, the mode of Si accumulation can be determined based on the relationship between DSi porewater concentrations and aboveground biomass BSi concentrations (Jones and Handreck, 1967; De Bakker et al., 1999; Norris and Hackney, 1999; Ma et al., 2001). In this case, a positive relationship between porewater DSi concentrations and above ground BSi concentrations indicates passive accumulation, a negative relationship indicates active accumulation, and no relationship indicates rejective accumulation (Raven, 1983; De Bakker et al., 1999).

While all vegetation contains some Si (Epstein, 1994), grasses (*Poaceae*) and sedges (*Cyperaceae*) typically accumulate the most (Jones and Handreck, 1967; Raven, 1983; Ma and Takahashi, 2002), which has been attributed to active Si accumulation by these plants (Jones and Handreck, 1967; Raven, 1983). However, it remains uncertain whether all wetland grasses fall into this category of active accumulation. For example, evidence exists that *Spartina* grasses, which are one of the most prolific genus of salt marsh grasses worldwide, are passive accumulators (Hou et al., 2010; Querné et al., 2012) and possibly even rejector plants (De Bakker et al., 1999).

Understanding the mechanism responsible for Si accumulation by salt marsh plants is important for understanding the controls on Si exchange at the land-sea interface. Tidal marshes, are large reservoirs of Si (Struyf et al., 2005b; Carey and Fulweiler, 2013), and have been shown to play a critical role in regulating Si availability in adjacent estuarine waters (Struyf et al., 2005a; Jacobs et al., 2008; Vieillard et al., 2011). This has important consequences for marine trophic structure (Officer and Ryther, 1980), as diatoms, a dominant type of phytoplankton in temperate coastal waters, require as much Si as N on a molar basis to survive (Redfield et al., 1963). Because the Si found in marsh plants (i.e., BSi) dissolves several orders of magnitude faster than mineral silicates (Alexandre et al., 1997; Cornelis et al., 2010a), understanding how marsh grasses sequester Si is a key step for understanding how salt marshes regulate Si exchange in these dynamic ecotones.

Currently, controls on Si accumulation in salt marsh grasses are not well understood. For example, in addition to uncertainties regarding the mode of Si accumulation (i.e., active, passive, or rejective accumulation), the role of plant age or growth rate in controlling Si accumulation remains unclear (Querné et al., 2012). Although increasing BSi content with growth has been observed for *Spartina* (De Bakker et al., 1999; Norris and Hackney, 1999; Querné et al., 2012; Carey and Fulweiler, 2013) and several species of freshwater marsh plants (Struyf et al., 2005b), Hou et al. (2010) found no increase in *S. alterniflora* BSi concentrations over the growing season. The highly dynamic nature of salt marshes, which experience a wide range in nutrient availability, sediment type, and hydrologic conditions, could be responsible for the non-uniform BSi concentrations found in salt marsh plants. Furthermore, the amount of Si in the surrounding environment may also exert an important control over plant Si accumulation, especially in cases of passive Si accumulation (Struyf et al., 2005b). In these cases, increased Si availability in soil solution, or porewater, would result in higher Si accumulation in the plants.

The primary objective of this study was to determine the drivers of BSi accumulation in *Spartina*, one of the most common types of salt marsh grasses found worldwide. We hypothesize that differences in *Spartina* Si accumulation are driven by environmental conditions and plant origin (native vs. non-native). To test this hypothesis, we used original data from five New England (USA) salt marshes, as well as published literature values, to examine the mode of Si accumulation, and the role of plant growth and *in situ* Si availability (in sediment and porewater) in controlling BSi accumulation in *Spartina*. We then developed a conceptual model which suggests that differences in aboveground BSi concentrations are due not only to differences in Si availability in marshes, but also different modes of Si uptake, representing a previously unrecognized form of phenotypic plasticity in these plants.

MATERIALS AND METHODS

We collected samples for Si accumulation at the height of growing season (spring) and during peak biomass (summer) in five marshes in New England, USA: a salt marsh in northern Maine (Site 1) and four Rhode Island (RI) salt marshes (Sites 2–5) (**Figure 1**, **Table 1**). All of the marshes displayed patterns of vegetation that are typical in New England marshes (Bricker-Urso et al., 1989; Wigand and Roman, 2012), with areas of "low marsh" that are inundated with tides twice a day consisting entirely of *Spartina alterniflora* grasses, while the areas of "high marsh" are inundated less frequently and are dominated by *S. patens* vegetation. Site 1 is a relatively undisturbed fringing salt marsh, located adjacent to Acadia National Park, draining an undeveloped watershed. The RI marshes cover a range of anthropogenic nutrient loadings and salinities (**Table 1**). Three of these marshes (Site 2–4) span the length of Narragansett Bay, from high to low nutrient inputs (DSi, N, P) (**Figure 1**, **Table 1**). Site 2 is a back-barrier marsh located on the east side of the Providence River Estuary, Site 3 is back-barrier marsh located on Prudence Island within the Narragansett Bay National Estuarine Research Reserve, and Site 4 is a fringing marsh located on the south side of Zeek's

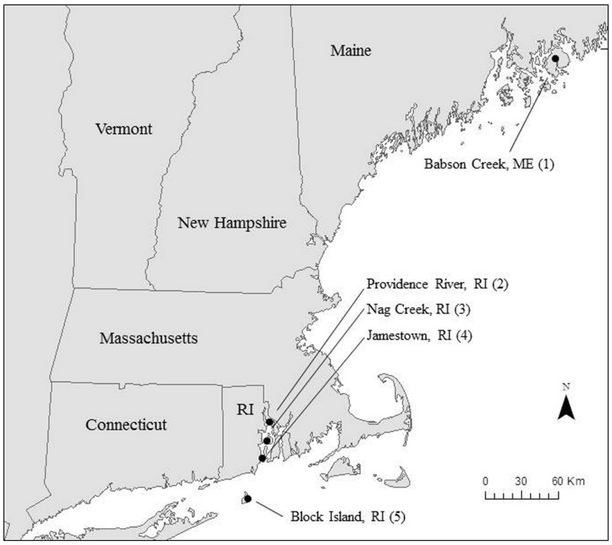

FIGURE 1 | Map of study region in New England, USA.

Table 1 | Site characteristics of estuaries adjacent to the salt marshes included in analysis.

Study and/or site name	Estuary	Salinity (ppt)	Bulk density (g cm^{-3})	DIN (μM)	DIP (μM)	DSi (μM)	Data source(s)
This Study							
1 (Babson Creek, ME)	Sommes Sound, USA	13.9 ± 2.7	0.70 ± 0.08	4.8 ± 0.2	0.23 ± <0.01	48 ± 6	Carey and Fulweiler, unpublished
2 (Little Mussachuck Creek)	Providence River Estuary, USA	27.7 ± 0.12	0.24 ± 0.04	4.5 ± 0.7	1.5 ± 0.11	19.6 ± 1.8	Krumholz, 2012 (nutrients)
3 (Nag Creek West)	Narragansett Bay, USA	30.0 ± 0.06	0.26 ± 0.03	1.5 ± 0.2	1.0 ± 0.08	16.7 ± 1.7	Krumholz, 2012 (nutrients)
4 (Zeek's Creek)	Narragansett Bay, USA	30.2 ± 0.12	0.25 ± 0.02	1.4 ± 0.1	0.73 ± 0.05	12.4 ± 1.0	Krumholz, 2012 (nutrients)
5 (Block Island)	Great Salt Pond, USA	29.2 ± 0.12	1.15 ± 0.04	6.2 ± 0.9	0.67 ± 0.11	na	URI Watershed Watch (www.uri.edu/ce/wq/ww) (nutrients)
Carey and Fulweiler, 2013 High-N (Apponaug)	Narragansett Bay, USA	30.1 ± 0.1	0.2 ± <0.01	22.3 ± 6	0.4 ± 0.12	45.6 ± 2.7	Carey and Fulweiler, unpublished
Querné et al., 2012	Bay of Brest, France	32–35	na	<5.6	<0.1	<1	Dauchez et al., 1991 (salinity) Ragueneau et al., 1994 (nutrients)
De Bakker et al., 1999	Oosterschelde, The Netherlands	25	0.3–0.6	3.5*, **	0.57**	4.5**	Nienhuis and Smaal, 1994 (nutrients) Oenema and DeLaune, 1988 (bulk density)
Norris and Hackney, 1999	Cape Fear River Estuary, USA	18.6	0.84 ± 0.07	9.3**	0.23**	63.8**	Freese, 2003, PhD dissertation (bulk density) Mallin et al., 1997 (nutrients, salinity)
Hou et al., 2010	Yangtze River Estuary, China	>20	1.3	22**	1.2**	28.6**	Yang et al., 2008 (Bulk density) Chai et al., 2009 (nutrients, salinity)

Unless otherwise noted, all values summer averages ± standard error.

**Does not include NH_{4+}, **annual average.*

Creek in Jamestown, RI (**Table 1**). Site 5 is fringing marsh in Great Salt Pond located 21 km south of the coast of RI, on a relatively undisturbed island (Block Island) exposed to low-nutrient ocean water.

We measured net Si accumulation in the aboveground vegetation, sediment, and porewater from the *S. alterniflora* zone at all sites. At sites 1 and 3, we also collected samples for aboveground vegetation, sediment, and porewater from the *S. patens* zone of the marshes. Sediment cores from sites 2–5 were also sieved for roots and rhizomes in order to analyze BSi content in belowground vegetation. In all cases, triplicate measurements of each of the parameters were sampled. Sampling was paired, so that in all cases where we sampled aboveground vegetation, samples for sediment, belowground vegetation, and porewater were also collected. Sediment cores and porewater were sampled from within the 0.25 m^2 plot where aboveground vegetation was collected. The same marshes were sampled during each season, but not the exact same 0.25 m^2 plot within each marsh, as our sampling was destructive (e.g., removal of grass and sediment from marsh). All samples from each individual marsh were collected on a single day. The triplicate values from each individual marsh were averaged for each season (spring and summer). These average values for spring and summer were treated as distinct value for all analyses.

We harvested above ground vegetation from three randomly chosen 0.25 m^2 plots per sampling event. Vegetation was washed thoroughly with deionized water and dried at 70°C for 48 h before it was ground using a Wiley Mill. We collected sediment cores using PVC corers (30 cm long, 5 cm diameter). We divided our cores into four sections—the top 1, 1–10, 10–20, and 20–30 cm. In order to calculate bulk density (mass of material per unit volume), we dried each core section at 70°C until it reached a constant mass over two consecutive days of weighing. Next, the cores were wet-sieved (0.5 mm sieve) to isolate roots and rhizomes, which were subsequently washed, dried, weighed, and analyzed for BSi concentrations. We collected triplicate porewater samples using porewater peepers, a passive sampling technique (Templer et al., 1998). We followed methods detailed in Carey and Fulweiler (2013), where 20-mL scintillation vials are pre-filled with deionized water and capped with a permeable membrane in order to allow the exchange of ions. Each polyvinyl chloride (PVC) peeper contained 10 vertically-stacked vials placed 3 cm apart. Peepers were deployed vertically in the sediment for 2–3 weeks, after which time the water was filtered using a 60-mL polypropylene syringe through a 0.45 micron nitrocellulose filter. The timing of peeper deployment always corresponded with collected of the other samples (e.g., sediment cores, aboveground vegetation). Porewater samples were stored in polyethylene bottles and kept in a cool dark place until they were analyzed for DSi. At sites 2–5, we also measured salinity and pH of all porewater samples.

We quantified sediment amorphous Si (ASi) content [which includes both BSi and pedogenic Si (Cornelis et al., 2010a)] and biomass BSi concentrations using the wet chemical alkaline extraction in 1% Na_2CO_3 solution (Demaster, 1981). Biomass was digested for 4 h. Sediment was digested for 5 h, with subsamples taken at hours 3 and 4 in order to calculate a mineral correction (Demaster, 1981; Conley and Schelske, 2001). We used a Seal AA3 flow injection analyzer and the molybdenum blue colorimetric method (Strickland and Parsons, 1968) to measure DSi concentrations. We routinely compared our standards to Hach external standards and they were always within 4% of the expected value.

In addition to our collected data from the five marshes in New England, we gathered all known published studies reporting aboveground plant tissue *Spartina* BSi concentrations and either productivity (biomass per unit area) and/or porewater DSi concentrations. In total, we located five studies that fit these characteristics (**Table 2**). The five studies presented data from salt marshes on three continents—Europe (i.e., the Netherlands, France), North America (i.e., New England and Southeast, USA), and Asia (i.e., China). We then identified general site characteristics of each site (e.g., bulk density and N availability) for comparison (**Table 1**).

In order to test the hypothesis that primary productivity or *in-situ* Si availability was driving aboveground BSi concentrations in *Spartina*, we ran correlation analysis between BSi concentrations in aboveground material and the following variables: primary productivity [a proxy for plant age for perennial grasses, which reach maximum productivity in August and senesces in the fall (Wigand et al., 2004)], ASi concentrations in the sediment, and DSi concentrations in porewater. We also ran simple linear regression and used the least squares method to estimate model parameters, with *Spartina* BSi concentrations as the independent variable in all cases. In addition to examining all data together to see if broad trends in *Spartina* BSi behavior were apparent, we grouped our data by species and region to determine any differences related to taxonomy or location. We used the slopes of the regressions between *Spartina* BSi concentrations and porewater DSi concentrations to identify the mode of Si accumulation in these plants, with strong positive slopes indicating passive accumulation, negative slopes indicating active accumulation and flat slopes indicating rejective accumulation. Comparisons across sites were determined using a One-Way ANOVA and "multcompare" command in Matlab. All statistics were done using Matlab using α of 0.05 as the threshold for significance. All BSi concentrations reported as percent SiO_2 by dry weight (dry wt.).

RESULTS

Si CONTENT OF NEW ENGLAND *S. ALTERNIFLORA*

A large range of aboveground BSi concentrations in *S. alterniflora* were found in the marshes, and concentrations typically increased throughout the growing season (**Table 3**). The minimum concentration observed was 0.24% by wt. (Site 5 in spring) and the maximum concentration measured was 1.04% by wt. (Site 2 in summer). These values align well with values reported in a meta-analysis by Hodson et al. (2005) and show that compared to other plant species in the order Poales, *Spartina* has relatively low Si concentrations.

Sediment ASi concentrations were almost always higher in the top 1 cm of the sediment compared to the depths of 1–30 cm (except Site 3 in spring) (Table S1). We found no significant differences in ASi concentrations seasonally or among sites, except for Site 5, which had ASi concentrations (0.35 ± 0.07%) an order of magnitude lower than all other sites in this study (Avg. 2.28 ± 0.47% by wt.) (**Table 3**, Table S1). Similar to the sediment ASi, the top one cm of the roots always had higher BSi content than the deeper depths (Table S2). Across all sites and both seasons, root BSi values ranged from a minimum of 0.18% observed at Site 5 in the summer to maximum value of 4.83% observed at Site 4 in summer. The average root BSi concentration across all sites and seasons was 1.03 ± 0.31%. Consistent with earlier observations in the same region (Carey and Fulweiler, 2013), the rhizomes always had significantly less BSi than the roots (Avg. 0.26 ± 0.03% by wt.) (Table S2). We found no seasonal or site related patterns to BSi concentrations in roots and rhizomes (Table S2).

Porewater DSi concentrations were higher during the summer compared to the spring and porewater values from Site 1 were typically higher than from the other marshes (**Table 4**, Table S3). Porewater concentrations ranged from 0 μM (top 1 cm at Site 2 in spring) to 384 μM (depth of 27 cm depth at Site 1 in summer) (Table S3). Across marshes, porewater pH was significantly ($p < 0.01$) different during both spring and summer seasons. For example, during the summer the pH at Sites 4 and 5 was significantly ($p < 0.01$) lower compared to Site 2.

Si CONTENT OF NEW ENGLAND *S. PATENS*

At two of the marshes in this study (Sites 1 and 3) we analyzed samples for Si accumulation in *S. patens*, or the high marsh platform zone of the marsh. Overall, Site 1 had higher Si accumulation than Site 3. For example, aboveground BSi concentrations of *S. patens* were roughly three times higher at Site 1 (**Table 3**), ranging from 0.58 to 1.25% by wt. (Site 2) and 0.21 to 0.43% (Site 3). Likewise, sediment ASi concentrations were also higher at Site 1 (Avg 2.16 ± 0.35% by wt.) than at Site 3 (Avg 1.31 ± 0.27% by wt.) (**Table 3**, Table S1). Similar to *S. alterniflora*, the ASi concentrations in the top one cm of sediment were always higher than the deeper layers of the core

Table 2 | Studies included in this analysis.

Study	Location	Species	Variables reported	Type
This study	New England, USA	*S. patens, S. alterniflora*	BSi, DSi, Productivity	Native
Carey and Fulweiler, 2013	New England, USA	*S. patens, S. alterniflora*	BSi, DSi, Productivity	Native
Norris and Hackney, 1999	North Carolina, USA	*S. alterniflora*	BSi, DSi, Productivity	Native
Querné et al., 2012	Bay of Brest, France	*S. alterniflora*	BSi, DSi, Productivity	Invasive
Hou et al., 2010	China	*S. alterniflora*	B Si, Productivity	Invasive
De Bakker et al., 1999	Netherlands	*S. anglica*	BSi, DSi	Invasive

Variables reported: DSi indicates porewater DSi (SiO_2) concentrations, BSi indicates aboveground tissue plant BSi concentrations. Productivity indicates biomass per unit area.

Table 3 | BSi concentrations in aboveground and belowground biomass, and ASi concentrations in sediment at each site.

Site	Aboveground (% BSi)		Belowground Biomass (% BSi)		Sediment (% ASi)
	Spring	Summer	Roots	Rhizomes	
S. alterniflora					
1	0.53 ± 0.06	0.95 ± 0.11	na	na	2.12 ± 0.49
2	0.48 ± 0.13	0.96 ± 0.05	1.04 ± 0.15	0.22 ± 0.04	3.76 ± 0.39
3	0.43 ± 0.07	0.60 ± 0.09	1.04 ± 0.17	0.24 ± 0.04	3.19 ± 0.44
4	0.30 ± 0.01	0.71 ± 0.13	1.39 ± 0.29	0.35 ± 0.07	2.83 ± 0.51
5	0.26 ± 0.02	0.45 ± 0.02	0.66 ± 0.11	0.23 ± 0.05	0.35 ± 0.08
S. patens					
1	1.01 ± 0.22	0.89 ± 0.14	na	na	2.16 ± 0.35
3	0.29 ± 0.07	0.31 ± 0.02	0.36 ± 0.04	0.13 ± 0.02	1.31 ± 0.27

Sediment values reported as ASi, as measurements include both biogenic and pedogenic Si. Where seasonal differences were observed (aboveground biomass only), we report both seasons separately. Otherwise, values represent average ± standard error across both seasons.

Table 4 | pH, salinity, and DSi concentrations (average ± standard error) measured in top 30 cm of porewater at each marsh site in this study.

site	Spring			Summer		
	pH	Salinity (ppt)	DSi (μM)	pH	Salinity (ppt)	DSi (μM)
S. alterniflora						
1	na	na	181.5 ± 33.3	na	na	239.5 ± 32
2	7.47 ± 0.09	24.0 ± 0.3	115.2 ± 18.3	7.07 ± 0.09	25.7 ± 0.4	156.3 ± 7.0
3	7.09 ± 0.02	29.6 ± 0.1	83.3 ± 4.1	7.22 ± 0.08	31.2 ± 0.4	121.7 ± 5.3
4	7.32 ± 0.07	30.1 ± 0.9	25.1 ± 3.3	6.42 ± 0.05	31.3 ± 0.4	55.4 ± 6.6
5	7.45 ± 0.03	30.0 ± 0.1	5.4 ± 1.1	6.60 ± 0.34	32.6 ± 0.3	55.1 ± 6.9
S. patens						
1	na	na	204.1 ± 39.4	na	na	153.0 ± 18
3	7.11 ± 0.07	28.3 ± 0.9	42.8 ± 5.6	7.3 ± 0.08	32.13 ± 1.1	49.5 ± 1.6

(except for summer Site 1 sample where 10–20 cm depth had slightly higher concentration) (Table S1). BSi in the roots and rhizomes in *S. patens* ranged from 0.08 to 0.96% and similar to *S. alterniflora*, BSi concentrations of the rhizomes (0.13 ± 0.02%) were much lower than in the roots (0.36 ± 0.04%) (Table S1).

Similar to other portions of the marsh budget, porewater concentrations were an order of magnitude higher at Site 1 than at Site 3 on all occasions (**Table 4**, Table S3). Average porewater salinity (30.2 ppt) and pH (7.21) at Site 3 under *S. patens* vegetation was similar to the values measured under *S. alterniflora* (**Table 4**).

BSi ACCUMULATION AS A FUNCTION OF PRODUCTIVITY

Using all available data from our analysis and the literature, we found a no correlation between aboveground productivity (mass per unit area) and BSi concentrations ($n = 31$, $R = 0.22$, $p = 0.23$) (**Figure 2**). We next subdivided the entire data by species

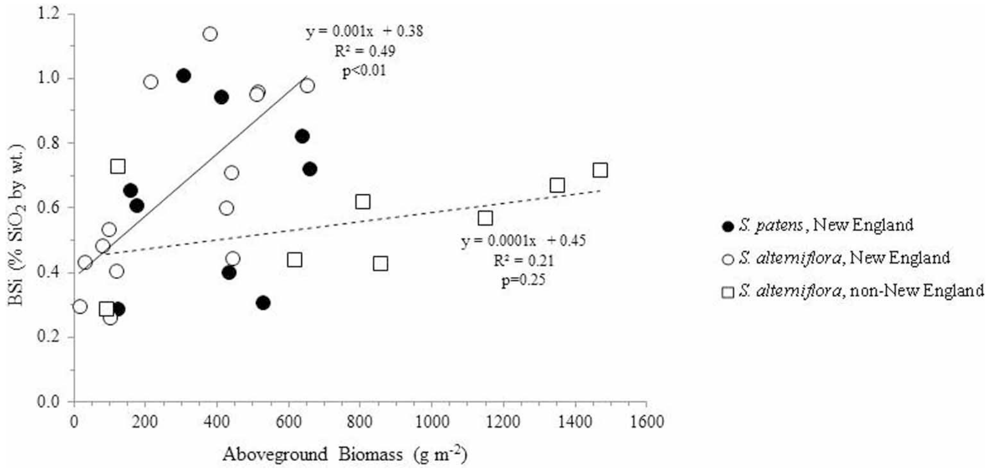

FIGURE 2 | BSi concentration in aboveground *Spartina* (*S. alterniflora* and *S. patens*) as a function of productivity. Using the entire dataset, no relationships were found in the data ($R^2 = 0.05$, $n = 31$). Similarly, no relationships between the variables were found in *S. patens* (filled circles) ($R^2 = 0.02$, $n = 9$) or non-New England *S. alterniflora* ($R^2 = 0.21$, $n = 8$) (dashed trend line). However, New England *S. alterniflora* BSi concentrations showed a significant ($p < 0.01$, $n = 14$) positive relationship with productivity ($R^2 = 0.49$) (solid trendline). In total these values represent nine marshes: three non-New England marshes (China, France, North Carolina, USA) and six New England marshes. New England data represent average values of triplicate field measurements, with separate points for spring and summer seasons. Individual points representing non-New England marshes either represent distinct seasons (Norris and Hackney, 1999; Hou et al., 2010) or marshes (Querné et al., 2012).

and by location in order to determine if regional or taxonomic differences resulted in distinct relationships between productivity and BSi accumulation in these plants. However, this continued to result in no significant relationships between productivity and BSi concentrations in most situations: *S. patens* ($n = 9$, $R = 0.14$, $p = 0.72$) and the non-New England *S. alterniflora* ($n = 8$, $R = 0.46$, $P = 0.25$). In fact, we only found the expected positive relationship between productivity and BSi concentration in New England *S. alterniflora* ($n = 14$, $R = 0.70$, $p < 0.01$) (this study, Carey and Fulweiler, 2013) (**Figure 2**). Although the ways in which BSi concentrations vary as a function of productivity is not uniform across sites, we found that all marshes, regardless of location, begin the growing season with a fairly consistent amount of BSi in *S. alterniflora* tissue (0.38 to 0.45% by wt.) (y-intercept, **Figure 2**). However, after the initial growing period, BSi accumulation diverges.

BSi ACCUMULATION AS A FUNCTION OF *IN SITU* Si AVAILABILITY

In order to test our hypothesis that *in situ* Si availability impacts BSi accumulation in aboveground plant tissue, we wanted to determine whether elevated sediment ASi concentrations or DSi in the porewater was associated with higher concentrations of aboveground BSi concentrations. Sediment ASi data was only available in conjunction with aboveground BSi concentrations for our original data, and for Carey and Fulweiler (2013). We found a strong correlation ($n = 22$, $R = 0.60$, $p < 0.01$) between sediment availability and BSi content (**Table 3**).

We found several studies, in addition to our own original data presented here, where porewater DSi concentrations are presented in conjunction with aboveground BSi concentrations (**Table 2**). Similar to the relationship between aboveground BSi content and productivity, examining all the data together resulted in no relationship between the concentrations of BSi in aboveground biomass and the concentration of DSi in the porewater ($n = 43$, $R = -0.20$, $p = 0.21$). We then subdivided the data by region and/or species in order to determine if there were differences based on taxonomy or origin. In doing so, different and yet, distinct relationships between porewater DSi and biomass BSi concentrations became apparent.

North American marshes

A positive correlation between *Spartina* BSi concentrations and porewater DSi concentrations ($n = 20$, $R = 0.77$, $p < 0.01$) (**Figure 3A**) was observed when all the data from New England was grouped together [excluding the data from the degraded "high-N" marsh described by Carey and Fulweiler (2013), which is discussed below]. In examining this regional grouping by species, we found a similar positive relationship for both *S. patens* ($n = 8$, $R = 0.93$, $p < 0.01$) and *S. alterniflora* ($n = 12$, $R = 0.72$, $p < 0.01$). However, data from one New England marsh [*S. alterniflora* at the N-enriched marsh ("high-N" site) presented by Carey and Fulweiler, 2013] showed a distinctly different pattern, as we observed a negative relationship ($n = 3$, $R = -0.98$, $p = 0.14$) between porewater DSi concentrations and BSi concentrations (**Figure 3C**).

Besides the data from the New England salt marshes, Norris, and Hackney completed a study of net Si accumulation in a mid-Atlantic marsh (North Carolina, USA). This study also observed a negative relationship between porewater DSi concentrations and *S. alterniflora* BSi concentrations ($n = 3$, $R = -0.94$, $p = 0.22$). However, unlike the pattern observed in the high-N marsh described by Carey and Fulweiler (2013), the data from this North Carolina marsh showed the opposite relationship with time, as over the course of growing season plant BSi concentrations decreased, while porewater concentrations increased (Figure S1).

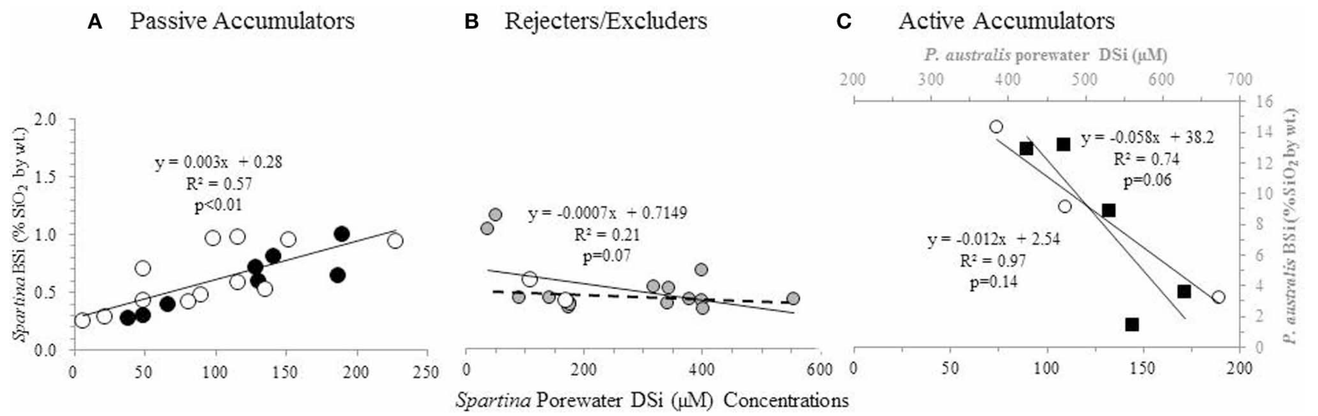

FIGURE 3 | BSi concentration in aboveground biomass as a function of porewater DSi concentrations. *S. patens* (black circles), *S. anglica* (filled gray circles), and *S. alterniflora* (open white circles), *P. australis* (black squares). Dashed line in **(B)** indicates the regression ignoring the two *S. anglica* outliers. Values in **(A,C)** represent average values from triplicate field measurements in top 20 cm of sediment, with spring and summer values plotted individually. Values in **(A)** represent values from five New England salt marshes. Values in **(B)** represent ten marshes in Oosterschelde, and the two marshes in Bay of Brest (Querné et al., 2012). Values in **(C)** represent values from one marsh in New England (*S. alterniflora* data) and one marsh in Belgium [*P. australis* data from Struyf et al. (2005b)].

European marshes

We observed a weak relationship between plant BSi and porewater DSi concentrations ($n = 18$, $R = -0.45$, $p = 0.07$) in the data from the European marshes (De Bakker et al., 1999; Querné et al., 2012). This weak negative relationship was primarily driven by two data points of low DSi porewater concentration (**Figure 3B**). Removing these two points (both of which are *S. anglica*) resulted in no relationship between those two variables ($n = 16$, $R = 0.07$, $p = 0.80$).

DISCUSSION

It is not uncommon for a single species of plant to contain different amounts of Si (Hodson et al., 2005), but such variability is typically thought to be due to differences in Si availability in the surrounding environment or growth rates (Norris and Hackney, 1999; Struyf et al., 2005b; Guntzer et al., 2012; Querné et al., 2012; Carey and Fulweiler, 2013). With regard to *Spartina*, productivity does not appear to be a major control over Si accumulation. Despite commencing the growing season with a relatively uniform amount of BSi, the relationship between productivity and aboveground BSi accumulation diverges at higher levels of productivity (**Figure 1**). This divergence indicates that productivity does not control BSi accumulation in the same manner in all *Spartina* species.

Differences in transpiration rates have been shown to be responsible for different levels of Si accumulation in plant leaves (Cornelis et al., 2010b; Guntzer et al., 2012). As such, the lack of relationship between productivity and BSi concentrations may be due to variable transpiration rates among salt marhes and/or water availability across these marshes, supporting the idea that productivity is not a good proxy for water use (Hessini et al., 2009). In addition to potential differences in water availability across sites, our results indicate that variable amounts of bioavailable Si present in the system and different modes of Si accumulation by the plants are the likely drivers of this pattern.

PASSIVE Si ACCUMULATION IN NEW ENGLAND MARSHES

In the New England marshes, higher ASi availability in the sediments is associated with higher BSi accumulation in the plants. This correlation is likely due to the soluble nature of ASi, which is considered a biologically available form of Si, having dissolution rates orders of magnitudes faster than mineral silicates (Struyf and Conley, 2012). New England salt marshes have some of the highest recorded sediment ASi concentrations (Carey and Fulweiler, 2013), which may explain why the BSi concentrations observed at several of these sites is higher than those found elsewhere (**Figure 1**).

Similar to the relationship observed with sediment ASi, higher porewater DSi concentrations are associated with higher aboveground BSi concentrations in all New England data, except in a highly degraded marsh (high-N marsh, Carey and Fulweiler, 2013) (discussed below) (**Figure 3A**). The positive relationship indicates that in the non-degraded New England marshes, *Spartina* passively accumulates Si, as more Si in the surrounding environment is associated with more Si in aboveground plant tissue. Thus, in this situation of passive accumulation, *in situ* Si availability in both the sediment and porewater are important controls Si accumulation in *Spartina*.

REJECTIVE Si ACCUMULATION IN EUROPEAN MARSHES

Unlike the strong positive and negative relationships we observed between plant BSi concentrations and porewater DSi concentrations in the New England marsh, we found no relationship between these two variables in the European marshes (**Figure 3B**). The lack of relationship between porewater DSi and plant BSi concentrations points toward *Spartina* in these systems behaving as a Si excluder, particularly at higher Si concentrations. In fact, De Bakker et al. (1999) also came to the same conclusion about their data, and suggested that *S. anglica* may reject Si from its tissue via a barrier on the outer surface of the roots, similar to legumes (Raven, 1983).

The extensive reviews by Jones and Handreck (1967) and Raven (1983) point toward rejector plants accumulating 5–60% of the DSi that is in the transpired water, with the remainder being left in the porewater. Further, at higher soil solution concentrations, plants will reject a larger portion of DSi (Raven, 1983; Cornelis et al., 2010b), which we also find evidence for with our flat regression line between aboveground BSi content and porewater DSi concentrations (**Figure 3B**). The exact mechanisms driving Si rejection in plants are not yet well understood, but it has been suggested that low Si accumulating plants have either a defective or non-existent Si transporter from cortical cells into the xylem (Ma and Yamaji, 2008).

Our designation of a Si excluder to *S. alterniflora* in the French salt marsh disagrees from the original interpretation of the data (Querné et al., 2012). While Querné et al. (2012) do point out the lack of relationship between DSi porewater and plant BSi concentrations, they assign *S. alterniflora* to the passive accumulator category based on the dry weight of BSi in the aboveground tissue alone. While this is a common practice when porewater DSi data is unavailable (Hou et al., 2010; Carey and Fulweiler, 2012), it ignores differences in transpiration or *in situ* Si availability. Thus, only defining Si accumulation by aboveground BSi concentration data may be misleading when passive Si accumulators are exposed to unusually high or low porewater concentrations, as one could incorrectly assign passive accumulators active or rejective status based on dry weight alone (Liang et al., 2005). Based on our analysis of the invasive *Spartina* from Europe, and the range of porewater concentrations observed in salt marshes, we are now skeptical at using the dry weight of BSi alone in quantifying mode of Si accumulation in marsh grasses.

ACTIVE Si ACCUMULATION IN DEGRADED MARSH

Unlike all other data from New England, we observed a negative relationship between porewater DSi availability and aboveground BSi content in the highly degraded salt marsh described in Carey and Fulweiler (2013) (**Figure 3C**). This negative relationship provides evidence of active Si accumulation, with the plants sequestering Si in high enough proportions to drive down porewater concentrations (Raven, 1983). As described by Raven (1983) and Jones and Handreck (1967), active Si accumulation results in the decline of DSi concentrations in the "bathing medium" as the plant takes up DSi relatively faster than it takes up water. Agricultural studies of active transport in rice, a known active Si accumulator, has identified two ATP-fuelled Si transporters (Ma and Yamaji, 2008), which transports Si through the membranes. Moreover, we consistently observed aboveground BSi concentrations >1% by wt., the threshold concentration shown to indicate active accumulation (Ma et al., 2001; Street-Perrott and Barker, 2008; Carey and Fulweiler, 2012; Querné et al., 2012).

In order to provide further support for our interpretation that the negative relationship between porewater DSi concentrations and aboveground BSi concentrations indicates active Si accumulation, we looked to Struyf et al. (2005b), who examined these two variables in a Western European tidal freshwater marsh dominated by *Phragmites australis*. *P. australis* is a known active Si accumulator, allowing us to examine the relationship between plant BSi and porewater DSi concentrations in a marsh experiencing active accumulation. Using the data presented by Struyf et al. (2005b), we found that *P. australis* also exhibits a strong negative relationship between porewater DSi concentrations and aboveground BSi concentrations ($n = 5$, $R = -0.86$, $p = 0.06$) (**Figure 3C**). The similar relationship between porewater and aboveground BSi concentrations in *P. australis* and the *S. alterniflora* in the degraded high N marsh provides additional evidence of active Si accumulation in the degraded marsh.

This site where evidence of active accumulation is observed is a high-nutrient, degraded salt marsh located downstream of an urban watershed (Wigand et al., 2003; Carey and Fulweiler, 2013). In fact, the N loading here is so high that the marsh is now P-limited, with a summer average molar DIN/DIP ratio in the inundating tidal creek water of 53 (**Table 1**). While it is possible that external factors are resulting in the declining porewater concentrations over time at this marsh, the fact that we do not observe this trend in the four nearby marshes indicates that the degraded, high N nature of the marsh is playing a role in the distinct behavior observed in *Spartina* here.

The results of one study, which focused on Si accumulation in a North Carolina (USA) salt marsh (Norris and Hackney, 1999), did not fit the criteria for any of the three modes of uptake. During our re-analysis of those data we observed a strong negative relationship between porewater DSi concentration and plant BSi concentration. While this trend initially indicates active accumulation, further examination of the change through time reveals that throughout the course of growing season, plant BSi concentrations decreased, while porewater concentrations increased (Figure S1). This is the opposite temporal relationship that one would find with active accumulation and does not fit the expected trend for passive or rejective accumulation either which signals a departure from any of the three traditional modes of uptake. The observations from this marsh call into question whether or not some plants, under certain conditions, can translocate Si.

ENVIRONMENTAL STRESS AND PHENOTYPIC PLASTICITY AS DRIVERS OF Si ACCUMULATION?

In examining the site characteristics of these marshes, we identify several variables that may drive the different modes of Si accumulation within marsh plants. The marshes studied here span a range of conditions, from nutrient over-enriched to relatively undisturbed, exposing these grasses to variable amounts of stress. Studies of abiotic stress on plant BSi concentrations report mixed results. Querné et al. (2012) found no plant response upon exposure to several types of stress, while Schoelynck et al. (2012) found increased Si accumulation in macrophytes exposed to intense water currents. A recent study of a degraded, "high-N" marsh found significantly more Si accumulation in several components of the marsh budget compared to a lower nutrient marsh (Carey and Fulweiler, 2013), which was hypothesized to be due in part to the stress induced by high air temperatures and low amounts of rainfall. We now propose that this degraded salt marsh is actively accumulating Si, which we suggest is a defense mechanism to ameliorate environmental stress.

In addition to marsh condition, genetic origin represents another variable across sites. *S. alterniflora* is native to the Atlantic and Gulf of Mexico coasts of North America, but invasive in many areas of the world, including the Pacific coast of North America, Europe, and Asia. Similarly, *S. anglica* is invasive in Western Europe. Invasive species in general have been shown to exhibit higher phenotypic plasticity than non-invasive species (Davidson et al., 2011). Phenotypic plasticity refers to the ability of organisms to change their observable traits in response to environmental conditions (Schlichting, 1986; Davidson et al., 2011). This phenomenon may be responsible for the different patterns in Si accumulation by salt marsh plants. Invasive species, particularly *S. alterniflora*, have been shown to exhibit higher phenotypic plasticity than the native plants (Zhao et al., 2010), which may explain the contrasting Si behavior by the same genus of plants. In particular, a recent study in China found the invasive *S. alterniflora* to show a higher degree of morphological growth, and biomass allocation plasticity in association with N availability compared to the native grass (Zhao et al., 2010). Our data suggests that genetic origin is another factor driving the differences in BSi accumulation in *Spartina* across sites. While phenotypic plasticity has been demonstrated for N, to our knowledge this is the first study to suggest a phenoplastic response of *Spartina* to Si.

A CONCEPTUAL MODEL DESCRIBING DRIVERS OF *Spartina* Si UPTAKE

Based upon the available data and our analysis above it is clear that wetland grasses cannot be grouped into one mode of Si accumulation. Rather we hypothesize that different Si accumulation modes are based on site-specific environmental conditions and genetic origin. To demonstrate this hypothesis we developed a conceptual model depicting the different modes of Si uptake by *Spartina* (**Figure 4**), which is based on the different slopes we observed in our linear regressions between porewater DSi availability and aboveground BSi concentrations (**Figure 3**). As illustrated in our model, we propose that *Spartina* passively accumulates Si in non-degraded marshes where the species are native, such as the case in the non-impaired New England marshes included in this study (**Figures 3A**, **4A**). Further, we suggest that *Spartina* behaves as Si excluders/rejectors in situations where the plant is invasive (**Figures 3B**, **4B**). Finally, we hypothesize that active Si accumulation by the native *Spartina* occurs when the plants are exposed to highly stressful, degraded conditions (**Figures 3C**, **4C**).

We present our conceptual model as a hypothesis which must be tested in future salt marsh research. Our hypothesis takes into account all known studies where *Spartina* BSi concentrations are reported alongside either porewater DSi concentrations or sediment ASi concentrations, but other confounding factors (e.g., diatom uptake, interaction with mineral silicates, and transport processes) also play a role in salt marsh Si dynamics. Thus, future work should focus on controlled field observations and laboratory studies which account for confounding variables as a means of testing the conceptual model that we put forth here. The recent recognition that gene expression can control Si accumulation in rice (Ma et al., 2006, 2007) highlights an exciting avenue of potential research for salt marsh grasses, such as *Spartina*.

While this is the first study to provide evidence of variable modes of accumulation in *Spartina*, Liang et al. (2005, 2007) observed both active and passive Si accumulation within several of the same species of plants (e.g., cucumber, rice, maize, sunflower, and wax gourd). Rather than the absolute categorization of "active" accumulator to grasses, the mode of Si accumulation within *Spartina* is variable depending on site-specific conditions. This interpretation aligns with the previous suggestion that plant species should be placed along a spectrum of accumulation, rather than categorized as having only one method of accumulation (Cooke and Leishman, 2011), an idea supported by recent work at the molecular-level of plant Si biochemistry (Ma et al., 2006, 2007; Ma and Yamaji, 2008).

ACTIVE Si ACCUMULATION AND A LOSS OF ECOSYSTEM SERVICE?

Tidal wetlands are important regulators of Si fluxes to adjacent estuarine systems (Struyf et al., 2005a; Jacobs et al., 2008; Vieillard et al., 2011), often supplying DSi necessary for diatom growth in

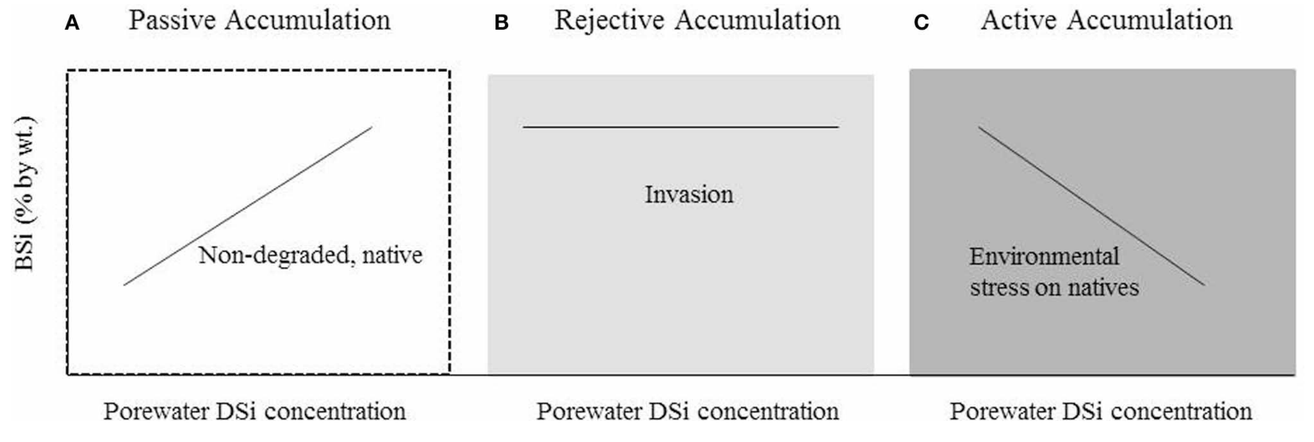

FIGURE 4 | Conceptual model hypothesizing how three modes of Si uptake in *Spartina* are related to site characteristics of those marshes, following the trends found in Figure 3. (A) Passive accumulation where native *Spartina* is found in non-degraded marshes, **(B)** Rejective accumulation where *Spartina* is invasive, **(C)** Active accumulation where native *Spartina* is exposed to environmental stress.

coastal systems (Anderson et al., 2002; Danielsson et al., 2008). A recent mass balance of Si fluxes in a New England salt marsh found the marsh to be a "point" source of DSi to the adjacent estuary, primarily due to drainage of Si-rich porewater during the ebbing tide (Vieillard et al., 2011). We observed a 60% decline in marsh porewater concentration over the growing season (spring through fall) in the marsh experiencing active Si accumulation. In turn, we hypothesize that the presence of active Si accumulators in marshes may diminish the export of DSi to downstream coastal systems by reducing DSi porewater concentrations. While the BSi sequestered in the plants will eventually be remineralized, this may occur after the plant has been exported from the marsh. Even in the case that BSi is remineralized *in situ*, it will ultimately shift the timing of DSi availability in the system. A shift to active Si accumulation by marsh grasses exposed to degraded conditions may represent a previously unrecognized way that coastal nutrient enrichment can impact estuarine ecology in North America.

Likewise *P. australis* is an invasive species throughout North American salt marshes (Chambers et al., 1999). We hypothesize that a shift in vegetation type from that of passively accumulating *Spartina* to actively accumulating *P. australis* could result in lower marsh porewater DSi concentrations. Such lower porewater DSi concentrations could reduce the ability for salt marshes to serve as sources of DSi to adjacent estuaries. In turn, we predict that invasion of *P. australis* to North American salt marshes may alter the timing and magnitude of Si exchange in coastal systems, highlighting a potentially unrealized consequence of this invasion to coastal ecosystems.

Tidal wetlands have the ability to control Si availability in coastal systems, which has important consequences for phytoplankton species composition. Here we present evidence that *Spartina* grasses may shift their mode of Si accumulation based on local environmental conditions and genetic origin. Recognition that these wetland grasses accumulate Si differently depending on site-specific characteristics provides new insight regarding the role these grasses play in Si dynamics at this borderland between land and sea.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00186/abstract

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Impact of rice cultivar and organ on elemental composition of phytoliths and the release of bio-available silicon

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The continental bio-cycling of silicon (Si) plays a key role in global Si cycle and as such partly controls global carbon (C) budget through nutrition of marine and terrestrial biota, accumulation of phytolith-occluded organic carbon (PhytOC) and weathering of silicate minerals. Despite the key role of elemental composition of phytoliths on their solubility in soils, the impact of plant cultivar and organ on the elemental composition of phytoliths in Si high-accumulator plants, such as rice (*Oryza sativa*) is not yet fully understood. Here we show that rice cultivar significantly impacts the elemental composition of phytoliths (Si, Al, Fe, and C) in different organs of the shoot system (grains, sheath, leaf and stem). The amount of occluded OC within phytoliths is affected by contents of Si, Al, and Fe in plants, while independent of the element composition of phytoliths. Our data document, for different cultivars, higher bio-available Si release from phytoliths of leaves and sheaths, which are characterized by higher enrichment with Al and Fe (i.e., lower Si/Al and Si/Fe ratios), compared to grains and stems. We indicate that phytolith solubility in soils may be controlled by rice cultivar and type of organs. Our results highlight that the role of the morphology, the hydration rate and the chemical composition in the solubility of phytoliths and the kinetic release of Si in soil solution needs to be studied further. This is central to a better understanding of the impact of soil amendment with different plant organs and cultivars on soil OC stock and on the delivery of dissolved Si as we show that sheath and leaf rice organs are both characterized by higher content of OC occluded in phytolith and higher phytolith solubility compared to grains and stems. Our study shows the importance of studying the impact of the agro-management on the evolution of sinks and sources of Si and C in soils used for Si-high accumulator plants.

Keywords: phytolith, rice, silicon solubility, PhytOC, soil-plant systems

INTRODUCTION

Silicon (Si), the second most abundant element in the continental surface (Wedepohl, 1995), is present in soils mostly as silicates, adsorbed Si onto oxides, biogenic precipitates called phytoliths and microorganisms remains. Dissolved Si (H_4SiO_4), can be readily taken up by plants and plays an important role as an alleviator of both biotic and abiotic stress (Epstein, 1994; Marschner, 1995; Mecfel et al., 2007). More importantly, Si is usually coupled with carbon (C) in different terrestrial biogeochemical processes (silicate weathering, soil formation, biota nutrition) that occur at different time-scales, which plays a crucial role in the regulation of atmospheric CO_2 (Oldenburg et al., 2008; Street-Perrott and Barker, 2008; Li et al., 2011; Song et al., 2012).

Si taken up by roots is mainly deposited in the transpiration sites of plants (e.g., cell walls, cell lumina, and intercellular spaces typically near evaporating surfaces) where polymerization of hydrated amorphous silica occurs to form phytoliths (Piperno, 1988; Ma, 2003). The chemical compositions of phytoliths consists mainly of SiO_2 (66–91%), organic carbon OC (1–6%), H_2O (0–11%), Al (0.01–4.55%), and Fe (0–2.1%) (Jones and Milne, 1963; Wilding, 1967; Wilding et al., 1967; Wang and Lü, 1993; Wang, 1998; Blecker et al., 2006). The chemical composition of phytoliths is highly dependent on plant species (Kameník et al., 2013). Despite their higher solubility compared to silicate minerals (Fraysse et al., 2009, 2010), phytoliths can be highly resistant to dissolution in specific physico-chemical conditions and can remain for thousands of years in soils (Wilding, 1967; Parr and Sullivan, 2005). Therefore, plant phytoliths play a major role in palaeobotanical, palaeoecological and archeological reconstructions (Clarke, 2003; Piperno, 2006; Cao et al., 2007; Raven and Giordano, 2009). Recent studies report that OC (0.2–5.8%) can be occluded in phytoliths (PhytOC) during plant growth (Parr and Sullivan, 2005; Parr et al., 2010; Li et al., 2013). Si polymerized in plant phytoliths may be more soluble compared to other crystalline mineral phases (Fraysse et al., 2009, 2010), while PhytOC is relatively more stable than other OC fractions in soils (Parr and Sullivan, 2005). For example, Parr and Sullivan (2005) report that the age of phytoliths in volcanic soils and peatland sediments ranges from 0 to 8000 year BP. Moreover, it has been reported that phytoliths in some sediments have a radiocarbon date of 13,300 ± 450 year BP (Wilding, 1967). PhytOC, a crucial component of terrestrial C sink, can represent up to 82% of total C in some soils and sediments after 2000 years of litter fall decomposition, contributing 15–37% in long term terrestrial C sequestration (Alexandre et al., 1997; Parr and Sullivan, 2005).

Cultivated rice (*Oryza sativa*) is the main food source for more than 50% of the global population (Salekdeh et al., 2002) and is currently cultivated on around 1.55×10^8 ha (i.e., 1.04% of the global terrestrial surface) (IRRI, 2011)[1]. Rice, a typical Si high-accumulator, can accumulate more than 10% of SiO_2 in dry matter (Alvarez and Datnoff, 2001) and application of Si may improve rice growth both under greenhouse and field conditions (Epstein, 1994, 1999; Liang et al., 2007). Recent studies showed that rice plays a significant role in the coupled terrestrial biogeochemical cycles of C and Si through the production of PhytOC (Parr et al., 2010; Song et al., 2012; Li et al., 2013). However, the impact of rice cultivar and of different rice organs on the phytolith elemental composition and solubility has never been reported (Savant et al., 1997), while solubility studies are important to make correct estimates of PhytOC preservation in soils and Si release in soil solution.

In this study, we investigated elemental compositions of organs and phytoliths, and the release of bio-available Si from rice phytoliths of different cultivars and organs (leaves, sheaths, stems and grains) cultivated in identical pedo-climatic conditions (Hortic Anthrosol in subtropical humid climate), to better understand the role of rice cultivars and organs on elemental composition (Si, Al, Fe, C) of phytolith and the release of bio-available Si (solubility in $CaCl_2$ solution at 0.01 M). Our results offer a research perspective to further study how to improve the phytolith C sequestration and Si fertility in soil-plant ecosystem.

MATERIALS AND METHODS

SAMPLING SITES

Organ samples (leaves, sheaths, stems and grains) of five rice cultivars (three replicates) were collected during the harvest season in October 2010, from the regional trials of new varieties of crops grown at the experimental site of Zhejiang Soil and Fertilizer Station (30°56′06.3″N and 120°51′52.9″E) in Jiaxing City, Zhejiang Province, southeast China. The experimental site is located in Hangjiahu Plain which experiences a typical subtropical humid monsoon climate, with an average annual precipitation of 1200 mm. The mean annual temperature is 16°C and the number of frost free days is 230. Soil is classified as the Gleysols (FAO, 1974)[2].

PLANT SAMPLE PREPARATION AND ANALYSIS

The five rice cultivars planted in the experiment were Jiahua-11, Xianghu-301, Zhejing-37, Ning-81, and Xiushui-09 which have been grown there for 8 years under identical pedo-climatic conditions to eliminate factors that might influence Si uptake and deposition (**Table 1**). We collected 8–10 rice cultivars from each site and their associated surface soil (0–5 cm). Then the stem, leaf, sheath and grains of each rice cultivar plant were separated. The sample material was thoroughly washed with ultrapure water after an ultrasonic bath for 15 min, dried at 75°C for 48 h. The dried and mixed samples were separated into two subsamples. One subsample was crushed to determine total elemental composition. Total Si, Al and Fe contents in plant samples and in a plant standard GBW 07602 (GSV-1) were determined by ICP-AES after combustion at 950°C followed by borate fusion (Lu, 2000).

The other subsample was cut into small pieces around 5 mm to extract phytoliths. Using a microwave digestion method (Parr and Sullivan, 2011) and Walkley-Black type digest (Walkley and Black, 1934), we extracted the phytoliths from all rice organs and thoroughly removed extraneous organic materials in the samples (Li et al., 2013). The phytoliths extracted were oven-dried at 75°C to a constant weight. The organic C released from phytoliths after HF treatment was dried at 45°C and the C content was determined by classical potassium dichromate method (Lu, 2000; Li et al., 2013). The organic C data were monitored with a standard reference soil of 30 mg (GBW07405). The precision is better than 7%.

Phytoliths samples were dissolved in an alkaline solution (NaOH, 1 M) (Saccone et al., 2006), in which the contents of Si, Al, and Fe were analyzed by ICP-AES.

BIO-AVAILABLE Si ANALYSIS

The pool of "bio-available Si" was determined by a $CaCl_2$ extraction (0.01 M) (Haysom and Chapman, 1975; Buck et al., 2010;

Table 1 | Physicochemical soil parameters in the soil surface (0–5 cm) for the different rice species cultivated in the experimental site.

Rice cultivars	pH	SOC	SiO_2	Fe_2O_3	Al_2O_3	Phytoliths
	g/kg					
Jiahua-11	5.8 ± 0.2a	23.4 ± 1.0b	661.1 ± 41.1a	279 ± 18.8a	142 ± 3.7a	19.2 ± 3.2a
Xianghu-301	5.8 ± 0.1a	15.2 ± 0.7c	681.4 ± 49.2a	286.5 ± 12.1a	146.2 ± 3.5a	11.6 ± 2.1b
Zhejing-37	6.0 ± 0.2a	24.8 ± 0.5b	660.6 ± 23.4a	271.9 ± 1.8a	147.1 ± 3.5a	15.3 ± 1.3ab
Ning-81	6.0 ± 0.0a	22.6 ± 0.4b	661.7 ± 37.4a	260.5 ± 11.3a	146.8 ± 2.8a	16.9 ± 2.1ab
Xiushui-09	5.8 ± 0.1a	29.3 ± 0.7a	670.2 ± 46.9a	281.2 ± 10.1a	146.10.5a	15.1 ± 1.0ab
Mean	**5.9a**	**22.9b**	**667.0a**	**275.8a**	**145.7a**	**15.6ab**

Means with various letters are significantly different at the $p < 0.05$ level of confidence according to Duncan's Multiple Range Test.

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[1] Available online at: http://ricestat.irri.org:8080/wrs2/entrypoint.htm.

[2] Available online at: http://en.wikipedia.org/wiki/FAO_soil_classification.

Narayanaswamy and Prakash, 2010) on dried phytolith samples. 30 mg of phytoliths was shaken in 50 ml of $CaCl_2$ solution at 20°C for 5 h, 1–9 days. At the end of each extraction, the concentration of Si was analyzed by ICP-AES. The amount of Si released in the extract over time represents the evolution of the immediately available Si fraction of the readily soluble Si pool (Berthelsen et al., 2001; Sauer et al., 2006; Cornelis et al., 2011). This kinetic extraction (expressed as mg bioavailable Si/g SiO_2 in phytoliths) has been designed to study the potential of phytoliths present in rice cultivars to replenish the bio-available Si pool (Houben et al., 2014).

SOIL SAMPLES PREPARATION AND ANALYSIS

Soil pH was measured in a 1:5 soil: water suspension. Soil organic carbon (SOC) was determined by wet digestion method with 133 mol L^{-1} $K_2Cr_2O_7$ and concentrated H_2SO_4 at 170–180°C. Total SiO_2, Fe_2O_3, and Al_2O_3 in soil were measured by ICP-AES after calcinations at 950°C followed by borate fusion (Lu, 2000). Soil phytolith extraction methods were slightly modified from methods of Piperno (1988) and Lu et al. (2006) as follows: $Na_4P_2O_7$ (20%) deflocculating, treatment with H_2O_2 (30%), and cold HCl (15%), $ZnBr_2$ heavy liquid (2.38 g-cm^3) separation. At the end of the soil extraction, the supernatant was separated from the solid residue by centrifugation (3000 g; 5 min) for analyzing phytolith content. All phytoliths extracted were oven-dried at 75°C and weighed to calculate their contents.

STATISTICAL ANALYSIS

All data are presented as the average of three analytical replicates (soil and plant samples). A One-Way analysis of variation (ANOVA) was carried out on the data obtained from the present study, and means were compared using Duncan's Multiple Range Test ($p < 0.05$). The statistical analyses were carried out with SAS software (SAS Institute, 1989).

RESULTS

PHYSICO-CHEMICAL SOIL CHARACTERISTICS

As shown in **Table 1**, the pH value (5.9) was similar in the soil surface of the five different rice species. The total concentration of Fe_2O_3 and Al_2O_3 were also identical in the soil surface of the five experimental plots, with mean value at 276 and 146 g/kg, respectively. The total concentration of SiO_2 was also identical in the soil surface of the five experimental plots, with mean value at 667 g/kg. The concentration of SOC and phytoliths varied from 15 to 29 g/kg, (mean at 23 g/kg) and from 12 to 19 g/kg (mean at 16 g/kg), respectively. This variation can be explained by the fact that the cultivation of different rice cultivars may cause differences in terms of straw production and residue input in soils, which can influence significantly the content of phytoliths and organic matter in soil.

PHYTOLITH CONTENT AND CHEMICAL COMPOSITION OF RICE ORGANS OF DIFFERENT CULTIVARS

The average content of phytoliths in the five rice cultivars varied significantly from 2.2 to 12.0% between rice organs (**Table 2**). Generally, the phytolith content decreased from 12.0% in sheaths >7.0% in leaves >3.7% in stems >2.2% in grains. The average of Al content for different organs in the five rice cultivars varied from 0.003 to 0.009% (sheaths > leaves = stems = grains). The Fe content in rice organs varied between 0.012 and 0.034% and showed the following trend: sheaths > stems = leaves = grains. The content of OC occluded in phytoliths (PhytOC) of rice organs varied significantly between 0.06 and 0.23% and showed the following trend: sheaths > leaves > stems > grains.

CHEMICAL COMPOSITION OF PHYTOLITHS EXTRACTED FROM DIFFERENT ORGANS

The mean H_2O content in phytoliths ranged from 5.4 to 14.5% and showed the following trend: stems > leaves > sheaths = grains. As a result of different hydration rates of phytoliths in the different organs, the SiO_2 content of phytolith was the highest in the grains (94.71%), then significantly decreased in sheaths (92.50%), leaves (88.47%), and stems (83.04%) (**Table 3**). The average Al content in phytoliths significantly varied from 0.012 to 0.043%: sheaths and leaves > grains and stems (**Table 3**; **Figure 1A**). The average Mg content in phytoliths varied from 0.0043 to 0.0082%: sheaths and leaves = grains > stems (**Table 3**). The average Fe content in phytoliths ranged from 0.017 to 0.009%: sheaths = leaves = grains = stems (**Table 3**; **Figure 1B**). The average OC content in phytoliths varied from 1.93 to 2.46%, without significant change between plant organs (**Table 3**).

THE RELEASE OF BIO-AVAILABLE Si

After 7 days, the cumulative release of bio-available Si from phytoliths of different plant organs (mg Si/g SiO_2 in phytoliths; **Figure 2A**) significantly varied from 7.8 to 44.9 mg Si/g SiO_2 and was the highest in leaves (44.9 mg Si/g of SiO_2), then significantly decreased in sheaths (18.1 mg Si/g of SiO_2), stems (12.2 mg Si/g of SiO_2), and grains (7.8 mg Si/g of SiO_2). After 7 days, the release of bio-available Si from phytoliths of stems of different rice cultivars (mg Si/g SiO_2 in phytoliths; **Figure 2B**) significantly varied from 6.9 to 15.7 mg Si/g SiO_2: Xiushui-09 (15.7 mg Si/g SiO_2) > Xianghu-301 (13.2 mg Si/g SiO_2) and Ning-81 (13.0 mg Si/g SiO_2) > Zhejiang-37 (7.5 mg Si/g SiO_2) and Jiahua11 (6.9 mg Si/g SiO_2).

DISCUSSION

IMPACT OF RICE CULTIVAR AND ORGAN ON CONCENTRATION AND ELEMENTAL COMPOSITION OF PHYTOLITHS

Our results show that a significant correlation exists between the phytolith content in plants and the elemental concentration in plants for the five rice cultivars (Phytolith vs. Si, $R^2 = 0.8895$; Phytolith vs. Al, $R^2 = 0.6494$; Phytolith vs. Fe, $R^2 = 0.572$) (**Table 4**). A correlation also exists between the phytolith content in plants and the phytolith elemental content (Phytolith vs. Phytolith-Al, $R^2 = 0.6518$; Phytolith vs. Phytolith-Fe, $R^2 = 0.2973$; Phytolith vs. Phytolith-Mg, $R^2 = 0.3194$) (**Table 5**). This implies that the uptake of Si Al, Fe may significantly affect phytolith production in plant organs. Previous studies already show that climatic (transpiration flow), pedologic conditions (Si availability) and plant species can influence significantly the uptake of Si and the formation of phytoliths in plants (Bartoli and Wilding, 1980; Epstein, 1999; Piperno et al., 2002; Hodson et al., 2005; Henriet et al., 2008; Cornelis et al., 2010, 2011; Kameník et al., 2013; Li et al., 2013). Our study

Table 2 | Elemental compositions (%) and phytolith content in organs (grains, sheath, stem and leaf) of the different rice cultivars.

Rice cultivars	Rice organs	Phytolith	Si	Al	Fe	PhytOC[a]
				%		
Xiushui-09	Grains	2.06 ± 0.03C	1.21 ± 0.15A	0.004 ± 0.001A	0.013 ± 0.002A	**0.04 ± 0.00AB**
Ning-81		2.66 ± 0.39A	0.98 ± 0.12A	0.003 ± 0.000AB	0.012 ± 0.000A	**0.08 ± 0.02A**
Xianghu-301		2.72 ± 0.58A	0.99 ± 0.02A	0.003 ± 0.000AB	0.012 ± 0.001A	**0.06 ± 0.02AB**
Zhejing-37		1.55 ± 0.58D	0.64 ± 0.06B	0.002 ± 0.001AB	0.013 ± 0.001A	**0.04 ± 0.02B**
Jiahua-11		2.16 ± 0.21B	1.26 ± 0.06A	0.001 ± 0.000B	0.009 ± 0.002A	**0.06 ± 0.01AB**
Mean		**2.23d**	**1.00d**	**0.003b**	**0.012b**	**0.06d**
Xiushui-09	Sheath	12.48 ± 0.57B	4.95 ± 0.09AB	0.012 ± 0.001A	0.030 ± 0.005B	**0.25 ± 0.02A**
Ning-81		10.89 ± 0.38D	4.37 ± 0.48B	0.011 ± 0.001A	0.047 ± 0.005A	**0.21 ± 0.01BC**
Xianghu-301		11.86 ± 0.58C	4.38 ± 0.21B	0.009 ± 0.001AB	0.033 ± 0.004B	**0.24 ± 0.02AB**
Zhejing-37		10.31 ± 0.24D	3.89 ± 0.38B	0.006 ± 0.001B	0.027 ± 0.002B	**0.23 ± 0.01ABC**
Jiahua-11		14.40 ± 0.70A	6.41 ± 0.79A	0.006 ± 0.002B	0.031 ± 0.001B	**0.20 ± 0.01C**
Mean		**11.99a**	**4.80a**	**0.009a**	**0.034a**	**0.23a**
Xiushui-09	Leaf	7.46 ± 0.01B	3.51 ± 0.32B	0.007 ± 0.002A	0.020 ± 0.004A	**0.14 ± 0.00B**
Ning-81		6.46 ± 0.92C	3.76 ± 0.25B	0.006 ± 0.001A	0.019 ± 0.003A	**0.16 ± 0.03B**
Xianghu-301		7.79 ± 0.52A	3.72 ± 0.23B	0.005 ± 0.001A	0.022 ± 0.001A	**0.17 ± 0.02AB**
Zhejing-37		7.93 ± 0.52A	3.37 ± 0.16B	0.004 ± 0.001A	0.022 ± 0.001A	**0.20 ± 0.02A**
Jiahua-11		7.46 ± 0.01AB	4.99 ± 0.35A	0.003 ± 0.001A	0.014 ± 0.001A	**0.17 ± 0.00AB**
Mean		**7.04b**	**3.87b**	**0.005b**	**0.019b**	**0.17b**
Xiushui-09	Stem	3.09 ± 0.34C	2.14 ± 0.07A	0.003 ± 0.000AB	0.029 ± 0.004A	**0.07 ± 0.01C**
Ning-81		3.74 ± 1.16B	1.26 ± 0.07A	0.005 ± 0.000A	0.012 ± 0.002B	**0.07 ± 0.03C**
Xianghu-301		3.91 ± 0.24AB	1.46 ± 0.17B	0.004 ± 0.000AB	0.029 ± 0.004A	**0.13 ± 0.01A**
Zhejing-37		4.02 ± 0.23A	2.14 ± 0.23B	0.004 ± 0.001AB	0.014 ± 0.000B	**0.09 ± 0.01BC**
Jiahua-11		4.00 ± 0.50A	2.08 ± 0.19A	0.002 ± 0.001B	0.014 ± 0.001B	**0.11 ± 0.02AB**
Mean		**3.75c**	**1.82c**	**0.004b**	**0.020b**	**0.09c**

Means with various letters are significantly different at the $p < 0.05$ level of confidence according to Duncan's Multiple Range Test.

Different lowercase and uppercase letters indicate significant differences among the stands in rice organs and rice cultivars.

[a] PhytOC, OC occluded in phytoliths per 100 g of dry matter for each organs.

in identical pedo-climatic conditions shows that the precipitation of elements between plant organs of rice cultivars is also controlled by plant physiology, i.e., more transpiration sites in leaves and sheaths. This explains why the elemental composition in organs is related to elemental composition in phytoliths.

Our study also demonstrates that for rice cultivars, a significant negative correlation exits between OC content of phytoliths and the elemental composition in plants (Phytolith-C vs. Phytolith, $R^2 = -0.3177$, Phytolith-C vs. Si, $R^2 = -0.312$, Phytolith-C vs. Al, $R^2 = -0.2656$) (**Table 5**). The higher the elemental content in plants is, the higher the phytolith content will be but the amount of occluded OC within the phytoliths will be lower. The different types of phytolith morphology and specific surface area between plant organs (e.g., between rice stem and rice sheath) may imply different occluded-OC content in phytoliths (Li et al., 2013). However, the impact of this factor on the OC occlusion within phytoliths remains to be examined.

THE PRODUCTION AND STABILITY OF RICE PHYTOLITHS

In the literature, we find some contradictory results about the control of plant species on the dissolution rate of phytoliths. Fraysse et al. (2009) document similar dissolution rates of phytoliths for four different plant species (horsetail, larch, elm and fern), while Bartoli and Wilding (1980) and Bartoli (1985) show that phytoliths from grass plants and deciduous species are more easily dissolved than that of coniferous species because of physico-chemical differences, i.e., higher Si/Al and Si/Fe ratios and water content. The adsorption of Al, Fe and other bi- and trivalent metals on phytoliths may influence their surface properties and decrease their rate of dissolution in the soil environment (Dove, 1995). In this study, the phytoliths of leaves and sheaths in five rice cultivars are characterized by lower ratio of Si/Fe and Si/Al compared to grains and stem (**Figures 1A,B**), which means that phytolith in leaves and sheaths incorporate more Al and Fe in their structure than in grains and stems. However, we clearly show that the kinetic release of bio-available Si from phytoliths in leaves are much higher relative to phytoliths from sheaths, grains and stems (**Figure 1A**). This is in contradiction with a previous view showing that phytolith solubility decreases with increasing metal content (Bartoli and Wilding, 1980; Bartoli, 1985; van Bennekom et al., 1989), but not with the results of Fraysse et al. (2009) showing similar dissolution rates for phytoliths with different elemental composition. This can be explained by the fact that phytolith solubility of rice cultivars could be controlled more by differences in terms of morphology (i.e., specific surface area) (**Figure 3**) and hydration rate (**Table 3**) than chemical composition. Our data show that the hydration rates of phytoliths (highest H_2O content in stem phytoliths) are not related to Si bioavailability which indicates that hydration rate can only explain a small part of the variation in phytolith solubility. This observation requires further investigations for better quantifying the impact of morphology and hydration rate on dissolution rates of phytoliths from different rice cultivars. The **Figure 1B** also shows that the release of bio-available Si from phytoliths in stems among different rice cultivars vary significantly. Thus, besides the impact of rice organs on phytolith solubility, we show that the genetics also partly governs the release of bio-available Si from phytoliths.

Phytoliths are a readily soluble, potential Si source for plants (Berthelsen et al., 2001; Sauer et al., 2006; Cornelis et al., 2011). Recent research also indicate that organic matter pyrolyzed from plants (biochar) with high phytolith content can be applied as a

Table 4 | Correlation analysis of the composition (elemental concentration and phytolith content) of organs in rice cultivars.

Elements	Phytolith	Si	Al	Fe
Phytolith	1	0.8895**	0.6494**	0.572**
Si		1	0.4612**	0.4249**
Al			1	0.4481**
Fe				1

***$p < 0.01$; ($n = 20$).*

Table 3 | Elemental composition (%) of phytoliths extracted from organs of different rice cultivars. Means with various letters are significantly different at the $p < 0.05$ level of confidence according to Duncan's Multiple Range Test.

Rice cultivars	Rice organs	SiO_2	Al	Mg	Fe	OC	H_2O^a
		%					
Xiushui-09	Grains	95.27 ± 1.93A	0.011 ± 0.007A	0.0050 ± 0.0004C	0.005 ± 0.001A	2.11 ± 0.12A	**2.58 ± 1.73AB**
Ning-81		95.08 ± 0.54A	0.011 ± 0.004A	0.0073 ± 0.0007AB	0.012 ± 0.001A	2.89 ± 0.84A	**1.98 ± 0.76B**
Xianghu-301		91.40 ± 1.02A	0.011 ± 0.003A	0.0058 ± 0.0003*B*C	0.007 ± 0.006A	2.35 ± 0.01A	**6.21 ± 1.44A**
Zhejing-37		95.74 ± 0.29A	0.022 ± 0.004A	0.0051 ± 0.0005C	0.014 ± 0.005A	2.32 ± 0.03A	**1.87 ± 0.41B**
Jiahua-11		96.07 ± 0.89A	0.016 ± 0.005A	0.0081 ± 0.0004A	0.018 ± 0.002A	2.62 ± 0.20A	**1.24 ± 1.26B**
Mean		**94.71a**	**0.014b**	**0.0063ab**	**0.011ab**	**2.46a**	**2.78c**
Xiushui-09	Sheath	93.16 ± 0.36A	0.048 ± 0.010A	0.0067 ± 0.0008B	0.014 ± 0.001A	2.03 ± 0.15A	**4.69 ± 0.51AB**
Ning-81		94.31 ± 0.15A	0.041 ± 0.002AB	0.0092 ± 0.0004A	0.012 ± 0.002A	1.96 ± 0.11A	**3.62 ± 0.21B**
Xianghu-301		91.34 ± 0.23A	0.028 ± 0.002B	0.0087 ± 0.0004A	0.013 ± 0.002A	2.05 ± 0.01A	**6.52 ± 0.33A**
Zhejing-37		92.31 ± 1.19A	0.055 ± 0.005A	0.0074 ± 0.0003AB	0.016 ± 0.002A	2.21 ± 0.11A	**5.34 ± 1.68AB**
Jiahua-11		91.40 ± 0.85B	0.043 ± 0.002AB	0.0091 ± 0.0004A	0.030 ± 0.001A	1.42 ± 0.17B	**7.04 ± 1.20A**
Mean		**92.50b**	**0.043a**	**0.0082a**	**0.017a**	**1.93a**	**5.44c**
Xiushui-09	Leaf	87.87 ± 2.50AB	0.036 ± 0.010A	0.0057 ± 0.0007B	0.016 ± 0.003AB	1.87 ± 0.31A	**10.16 ± 3.54AB**
Ning-81		93.54 ± 0.98A	0.052 ± 0.003A	0.0105 ± 0.0008A	0.022 ± 0.002A	2.45 ± 0.14A	**3.86 ± 1.39B**
Xianghu-301		83.22 ± 0.84B	0.043 ± 0.002A	0.0092 ± 0.0004A	0.011 ± 0.001A	2.21 ± 0.04A	**14.46 ± 1.19A**
Zhejing-37		87.83 ± 1.87AB	0.034 ± 0.009A	0.0050 ± 0.0004B	0.012 ± 0.003AB	2.57 ± 0.15A	**9.51 ± 2.64AB**
Jiahua-11		89.87 ± 2.43AB	0.034 ± 0.004A	0.0042 ± 0.0004B	0.009 ± 0.004B	2.30 ± 0.04A	**7.75 ± 3.44AB**
Mean		**88.47c**	**0.040 a**	**0.0069a**	**0.014ab**	**2.28a**	**9.15b**
Xiushui-09	Stem	80.22 ± 1.63C	0.009 ± 0.005A	0.0053 ± 0.0001A	0.005 ± 0.002B	2.11 ± 0.12C	**17.64 ± 2.31A**
Ning-81		82.14 ± 2.75*B*C	0.013 ± 0.004A	0.0047 ± 0.0003B	0.010 ± 0.001AB	1.85 ± 0.16C	**15.96 ± 3.89AB**
Xianghu-301		80.75 ± 0.52*B*C	0.013 ± 0.002A	0.0041 ± 0.0004B	0.010 ± 0.001AB	3.36 ± 0.04A	**15.84 ± 0.74AB**
Zhejing-37		84.74 ± 1.68AB	0.009 ± 0.005A	0.0037 ± 0.0004B	0.007 ± 0.001AB	2.21 ± 0.11C	**13.02 ± 2.38AB**
Jiahua-11		87.37 ± 0.12A	0.015 ± 0.005A	0.0039 ± 0.0004B	0.012 ± 0.002A	2.73 ± 0.15B	**9.85 ± 0.17B**
Mean		**83.04c**	**0.012b**	**0.0043b**	**0.009b**	**2.45a**	**14.46a**

Different lower case and uppercase letters indicate significant differences among the stands in rice organs and rice cultivars.

[a] *According to the following equation to estimate the content of H_2O in phytoliths. $H_2O = 100\text{-}SiO_2\text{-}Al_2O_3\text{-}Fe_2O_3\text{-}MgO\text{-}OC$.*

Table 5 | The correlation between phytolith elements and plant elements.

plant elements Phytolith elements	Phytolith-Si	Phytolith-C	Phytolith-Al	Phytolith-Fe	Phytolith-Mg
Phytolith	0.0158	−0.3177**	0.6518**	0.2973**	0.3194**
Si	0.0056	−0.312**	0.6809**	0.3128**	0.254*
Al	0.0223	−0.2656*	0.4069**	0.0731	0.2311*
Fe	0.0044	−0.1153	0.2711*	0.061	0.1953*

$^{*}p < 0.05$; $^{**}p < 0.01$; ($n = 20$).

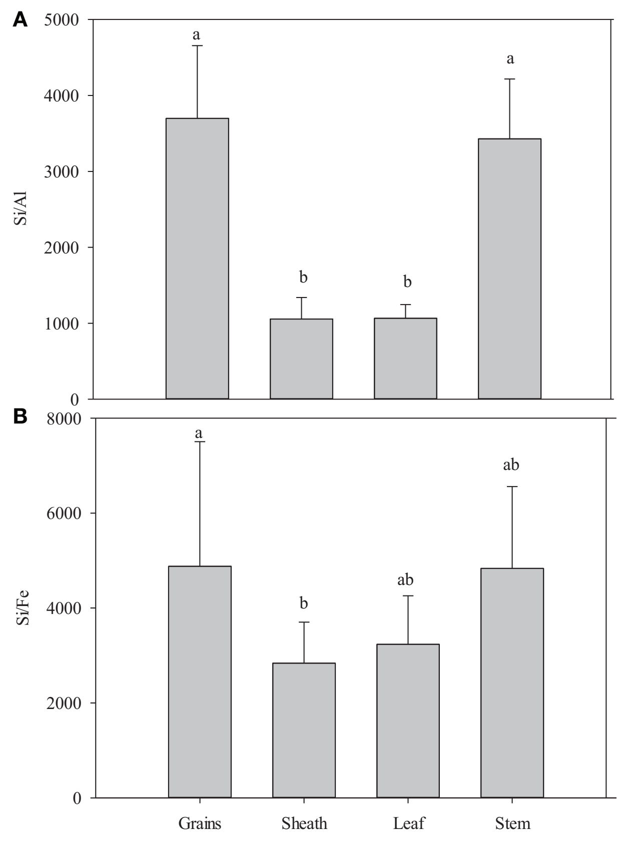

FIGURE 1 | The ratio of Si/Al (A) and Si/Fe (B) in phytoliths of five rice cultivars. Means with various letters are significantly different at the $p <$ 0.05 level of confidence according to Duncan's Multiple Range Test.

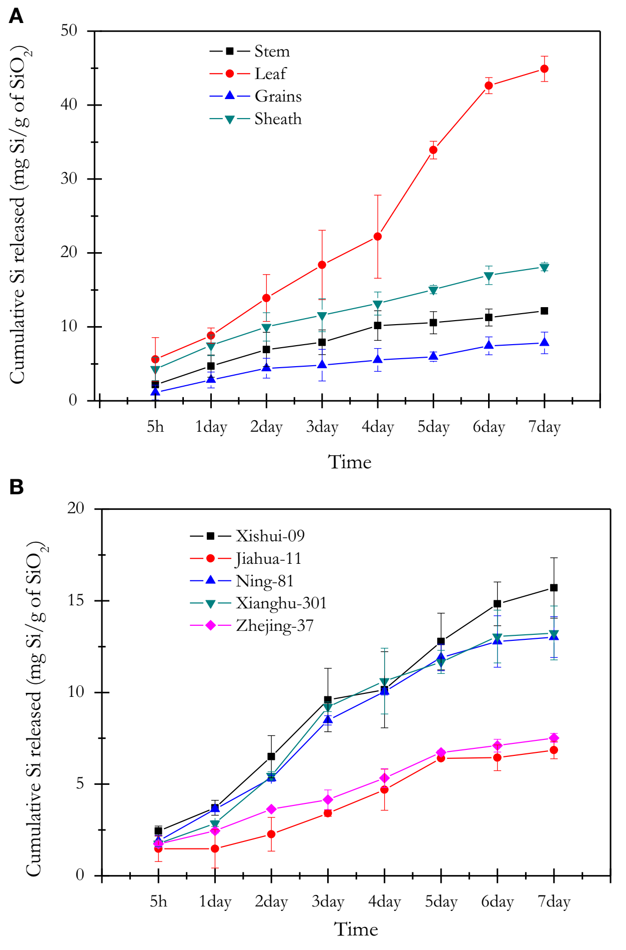

FIGURE 2 | Cumulative amount of Si released over time per g of SiO_2 in phytoliths (mg bioavailable Si/g SiO_2 in phytoliths) by successive extractions with 0.01 M $CaCl_2$. (A) Si released from phytoliths extracted from the organs of five rice cultivars (each phytolith sample is the mixture of same organ from five rice cultivars); **(B)** Si released from phytoliths extracted from stems of five rice cultivars. Each point represents the mean of three replicates.

potential source of bio-available Si for crops of Si-accumulator plants (Houben et al., 2014; Liu et al., 2014). Therefore, amendment with biochar made from leaves and sheaths of rice characterized by the highest amount of phytoliths and the highest release rate of Si could be promising for enhancing the bio-availability of Si while increasing OC storage and soil fertility.

The findings of this study suggest that the optimization of utilization of different plant organs as Si amendment could play a key role for more efficient productivity in high Si-accumulating plants. On the other hand, we should increase PhytOC content in soils by increasing return of plant organs characterized by lower phytolith solubility such as grains and stems. We thus suggest further study of the kinetic release of dissolved Si and dissolved OC and the evolution of Si and OC stock in soils after amendment with different plant organs.

CONCLUSIONS

The present study mainly focused on the elemental compositions of organs and phytoliths in rice of different cultivars. The

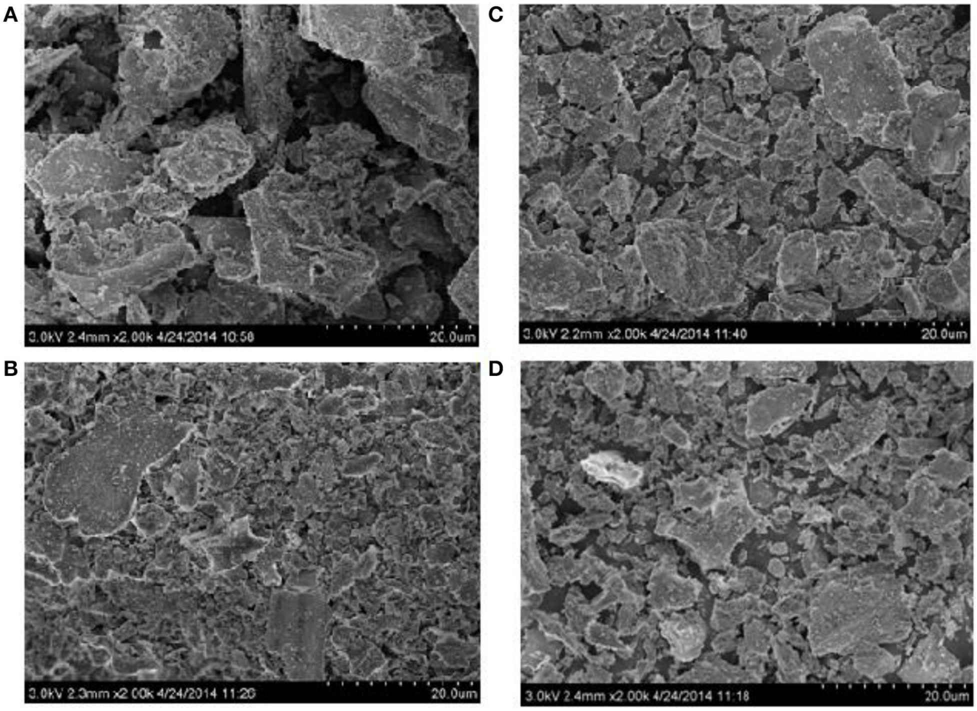

FIGURE 3 | Representative scanning electron microscope (SEM) images of phytoliths from rice organs (A) grains, (B) leaf, (C) sheath, and (D) stem.

rice cultivar significantly influences the elemental composition of plant organs and phytoliths. Our results show that rice cultivar and Si, Al, Fe uptake may impact production of phytoliths in plants and their quality (chemical composition). The OC content of phytoliths in rice cultivars seems to be significantly affected by the content of Si, Al, and Fe in plants, but not by the elemental concentrations in phytoliths.

In different rice cultivars, the role of the morphology and hydration rate of phytoliths seems to be at least as important as the chemical composition. This documents that rice organs and cultivar can largely influence Si dynamics in soil-plant systems through variable solubility of phytoliths. The soil amendment with plant residues and/or plant biochar should therefore be carried out taking into account the phytolith solubility of different plant organs of Si-high accumulator plants (e.g., leaves and sheaths of rice). On the other hand, phytoliths from grains and stems of rice seem to be more stable in soil and should be used if we aim to increase the soil OC stock through higher PhytOC. A more efficient use of plant residue (type of plant organ) as soil amendment should be considered to improve agronomical productivity of Si-high accumulating plants.

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Does elevated CO_2 alter silica uptake in trees?

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Human activities have greatly altered global carbon (C) and Nitrogen (N) cycling. In fact, atmospheric concentrations of carbon dioxide (CO_2) have increased 40% over the last century and the amount of N cycling in the biosphere has more than doubled. In an effort to understand how plants will respond to continued global CO_2 fertilization, long-term free-air CO_2 enrichment experiments have been conducted at sites around the globe. Here we examine how atmospheric CO_2 enrichment and N fertilization affects the uptake of silicon (Si) in the Duke Forest, North Carolina, a stand dominated by *Pinus taeda* (loblolly pine), and five hardwood species. Specifically, we measured foliar biogenic silica concentrations in five deciduous and one coniferous species across three treatments: CO_2 enrichment, N enrichment, and N and CO_2 enrichment. We found no consistent trends in foliar Si concentration under elevated CO_2, N fertilization, or combined elevated CO_2 and N fertilization. However, two-thirds of the tree species studied here have Si foliar concentrations greater than well-known Si accumulators, such as grasses. Based on net primary production values and aboveground Si concentrations in these trees, we calculated forest Si uptake rates under control and elevated CO_2 concentrations. Due largely to increased primary production, elevated CO_2 enhanced the magnitude of Si uptake between 20 and 26%, likely intensifying the terrestrial silica pump. This uptake of Si by forests has important implications for Si export from terrestrial systems, with the potential to impact C sequestration and higher trophic levels in downstream ecosystems.

Keywords: elevated CO_2, silicon, forest Si uptake, terrestrial Si pump, active Si accumulation, Si cycling

INTRODUCTION

We are currently conducting a global experiment by exposing Earth's biosphere to atmospheric carbon dioxide (CO_2) concentrations unseen since the early Miocene, some 23 million years ago (Pearson and Palmer, 2000). From the start of the Industrial Revolution we have increased CO_2 concentrations by approximately 40% (Pearson and Palmer, 2000) and in the spring of 2014 CO_2 levels officially exceeded 400 ppm at the Mauna Loa Observatory (NOAA, 2014). This grand experiment will continue for the foreseeable future, as over the next century CO_2 concentrations are expected to increase further (IPCC, 2013).

The impact of increased CO_2 concentrations on the terrestrial biosphere has received much research attention over the last several decades. In particular, recent research has focused on how plants have and will respond to rapid CO_2 concentration increases in combination with other regional and global climate changes, including warming air temperature and increased N availability (Norby and Luo, 2004; Ainsworth and Long, 2005; Bernhardt et al., 2006; Finzi et al., 2006). Among numerous other impacts, CO_2 enrichment can also alter plant stoichiometry (Loladze, 2002). Exposure to elevated CO_2 concentrations can cause declines in leaf nutrients, such as N and phosphorus, as well as trace element concentrations (Taub et al., 2008). The mechanism driving this decline is unclear but it has been attributed to nutrient limitation, increased non-structural carbohydrates, lower transpiration rates, or changes in nutrient allocation patterns (Roberntz and Linder, 1999). A compilation of studies on herbaceous and woody plants found that elevated CO_2 concentrations decreased foliar element concentrations by up to 15% (Loladze, 2002). Research on rice, a key global crop, found similar declines in essential elements such as N, magnesium, and iron. Such declines in plant elemental concentrations could have serious repercussions for higher trophic levels, including exacerbation of human malnutrition (Loladze, 2002). A meta-analysis by Cotrufo et al. (1998a) found that aboveground N content declined on average by 14% under high CO_2 concentrations. Changes in the N content of litter have also been shown to alter rates of decomposition and thus nutrient cycling within terrestrial ecosystems (Cotrufo et al., 1998b). However, changes in foliar elemental composition could also have large-scale impacts on the cycling and transport of nutrients from land to the sea.

Silicon (Si) is the seventh-most-abundant element in the universe and the second-most abundant element in soils, the mineral substrate for most of terrestrial plant life (Epstein, 1994; Tréguer and De La Rocha, 2013). In the ocean, Si is a key nutrient required for diatoms and is used by many species of sponges, radiolarians, silicoflagellates, choanoflagellates, and even picocyanobacteria (Baines et al., 2012). Of particular importance are diatoms, as they form the base of many productive marine food webs and they sequester significant amounts of C to the deep ocean. In fact, a recent modeling effort contributes 50% of global ocean productivity to diatoms (Rousseaux and Gregg, 2013). The primary source of Si to the ocean is the transport of dissolved and biogenic silica (BSi; SiO_2) via rivers, which together account for 78% of the net annual Si oceanic inputs (Tréguer and De La Rocha, 2013). The Si transported by rivers ultimately comes from the weathering of the lithosphere, which is dependent on complex interactions between climate, geology, and biology (Bluth and Kump, 1994; Conley, 2002; Derry et al., 2005). Recently there has been emphasis on the role of biology in altering the timing and magnitude of Si export, specifically in terms of biological uptake by terrestrial vegetation (Conley, 2002; Fulweiler and Nixon, 2005; Gérard et al., 2008; Carey and Fulweiler, 2013b) and the role of human activities in directly altering watershed Si export (Clymans et al., 2011; Carey and Fulweiler, 2012a, 2013b; Vandevenne et al., 2012).

Plants readily absorb dissolved silica (DSi), also known as silicic acid (H_4SiO_4), the dominant form of Si in soil solutions (Epstein, 1994). DSi is taken up with water and carried in the transpiration stream where, with the evaporation of water, it becomes supersaturated and precipitated as BSi or phytoliths (Raven, 2003). Si provides numerous benefits to vegetation including increased resistance to bacteria, fungi, and grazers, as well protection from desiccation and metal toxicity (Hodson and Evans, 1995; Epstein, 1999; Wieczorek et al., 2014). Si is found throughout plants, from their roots to their shoots, but peak concentrations are generally observed at the transpiration termini (Canny, 1990). In fact, Si can compose 10% or more of the dry weight, exceeding those concentrations of well-known macronutrients (i.e., N and potassium; Epstein, 1994). In turn, the accumulation of Si by terrestrial vegetation over the seasonal cycle has the capacity to regulate the watershed export of Si to coastal ecosystems as plants grow and senesce (Fulweiler and Nixon, 2005; Carey and Fulweiler, 2013b). Alternatively, because BSi is 7–20 times more soluble than mineral silicates (Cornelis et al., 2010a), plants may also provide an important source of Si on biological times scales.

Within this context we wanted to determine if trees exposed to elevated CO_2 concentrations would exhibit a decline in foliar Si content like those observed for other elements. To do this, we analyzed BSi concentrations of leaf samples from coniferous and deciduous trees from the Duke free-air CO_2 enrichment (FACE) experiments in North Carolina. Additionally, we examined Si content under nitrogen (N) enrichment, as well as under the combined impact of CO_2 and N enrichment. This is the first study to specifically examine the role of CO_2 and N enrichment on Si content in trees.

MATERIALS AND METHODS

The Duke Face experiment is located in a *Pinus taeda* L. (*P. taeda*, loblolly pine) plantation at Duke University in North Carolina (35°58′N, 79°06′W). This plantation was established in 1983 and is characterized as having moderately low-fertility and acidic clay loam (McCarthy et al., 2010). In addition to the dominant pine, deciduous species present include *Acer rubrum* (*A. rubrum*, red maple), *Cercis canadensis* (*C. Canadensis*, red bud), *Cornus florida* (*C. florida*, dogwood), *Liquidambar styraciflua* (*L. styraciflua*, sweet gum), and *Ulmus alata* (*U. alata*, winged Elm). Mean annual precipitation is 1145 mm.

This study was conducted on leaves collected from CO_2 and N enrichment experiments that took place between 1996 and 2006. The technical details of these studies have been published previously (e.g., Andrews et al., 2000; Luo et al., 2003; Finzi et al., 2007; McCarthy et al., 2010). Briefly, triplicate 30 m diameter treatment plots were exposed to current +200 ppm of CO_2 above ambient during daylight hours in the growing season. Control plots ($n = 3$) were treated in a similar manner but with the addition of ambient air instead of CO_2 (McCarthy et al., 2010). In 1998, two of these plots were divided in half and N fertilization began (11.2 g N m^{-2} y^{-1} as urea). For more detailed information on the experimental design see http://face.env.duke.edu.

For this analysis we used dried green leaf samples from *P. taeda* and the five broadleaf species listed above, collected in 2002–2003 and 2006, respectively. *P. taeda* samples ($n = 31$) were separated and ground into a powder using a mortar and pestle. The premilled deciduous samples ($n = 98$) each contained composites of several individuals.

Biogenic Si concentrations were determined using a wet alkaline chemical extraction in a 1% Na_2CO_3 solution (Demaster, 1981; Conley and Schelske, 2001). Duplicate samples were weighed to approximately 30 mg (between 28 and 34 mg) and digested in flat bottomed polyethylene bottles in a shaker bath at 85°C and 100 rpm for four hours. We used a Seal AA3 flow injection autoanalyzer to colorimetrically determine DSi from the BSi aliquots using the molybdenum blue colorimetric method (Strickland and Parsons, 1972). Standards made of sodium hexafluorosilicate (Na_2SiF_6) as well as external standards were used throughout the analysis to check accuracy and were always within 4% of the expected value. We report all BSi values as %Si by dry weight.

All statistical analyses were completed using JMP Pro 10.0 and significance was judged with an alpha of 0.05. BSi concentrations across species and treatments exhibited equal variances according to several commonly used unequal variance tests (O'Brien = 0.6385, Brown-Forsythe = 0.3196, Levene = 0.1978). To explore potential drivers of BSi concentrations we used a linear mixed effects model to address if the BSi concentrations were different across species and treatment alone and combined. In this model we treated station as a random effect to assess the potential random station effects of the block design used at the Duke FACE experiment. We used an ANOVA to further explore differences in BSi concentrations across species and followed it by a *post hoc* means comparison with Tukey's test for honestly significant differences (HSD).

RESULTS

Across all sample types and treatments BSi values ranged from 0.05 to 3.01 %Si dry wt., with a median of 0.82 %Si dry wt. and mean of 0.98 %Si dry wt. *C. florida* exhibited the lowest and least variable BSi concentrations, while *U. alata* had the highest (**Table 1**; **Figure 1**). While some species did exhibit a decline in foliar BSi concentrations under elevated CO_2, we found no statistically significant effect of treatment on foliar BSi concentrations (**Table 1**). In fact, our least squared model showed no effect of treatments, station, or treatment by species.

Because of the lack of statistical difference in plant BSi concentrations across all experimental treatments, we averaged

Table 1 | The mean foliar biogenic silica (BSi) concentrations (%Si by dry wt. ± SE) under the three treatment types and the control for five deciduous and one coniferous species at the Duke FACE experimental forest.

	Treatment			
Species	**Control**	**CO_2 enrichment**	**N enrichment**	**N and CO_2 enrichment**
	(%BSi as Si dry wt.)			
Cornus florida (dogwood)	0.07 (±0.01)	0.07 (±0.01)	0.07 (±0.01)	0.08 (±0.01)
Pinus taeda (loblolly pine)	0.97 (±0.07)	0.90 (±0.36)	nm	nm
Cercis canadensis (red bud)	0.27 (±0.02)	0.19 (±0.00)	0.16 (±0.02)	0.50 (±0.21)
Acer rubrum (red maple)	1.06 (±0.12)	0.91 (±0.08)	0.98 (±0.19)	0.94 (±0.06)
Liquidambar styraciflua (sweet gum)	1.08 (±0.16)	0.95 (±0.05)	0.72 (±0.03)	0.92 (±0.11)
Ulmus alata (winged elm)	2.36 (±0.16)	2.57 (±0.23)	2.17 (±0.11)	1.90 (±0.35)

nm = not measured.

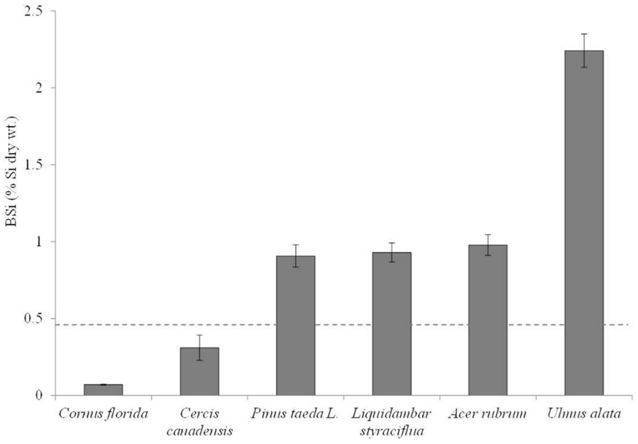

FIGURE 1 | Mean BSi concentrations (as %Si by dry wt.) in ascending order for the species analyzed in this study. The most simplistic definition of active Si accumulation in plants is defined as above ground %Si > 0.46 which is shown here with the dashed line.

BSi concentrations by species. Clear species differences exist (**Figure 1**), as the majority of species are statistically different from one another ($p < 0.0001$). The exceptions are that *P. taeda*, *A. rubrum*, and *L. styraciflua* are not statistically different from each other, but they are statistically different from *C. florida*, *C. canadensis*, and *U. alata*. *U. alata* exhibited BSi concentrations statistically higher than all other species ($p < 0.0001$). In all cases, except for *C.* and *C. canadensis*, the mean Si foliar content suggests active accumulation (i.e., %Si above 0.46%; **Figure 1**).

DISCUSSION

In this study we examined foliar BSi concentrations in five deciduous and one coniferous species across three treatments: CO_2 enrichment, N enrichment, and N and CO_2 enrichment. We expected to see a decrease in Si content as CO_2 increased, compared to the control group, as this phenomenon has been observed for a range of other elements such as N, phosphorus, iron, and zinc (Loladze, 2002). In four species (*P. taeda, C. canadensis, A. rubrum, L. styraciflua*) we did observe a decline in BSi concentrations under elevated CO_2 but none of them were statistically significant (**Table 1**). The largest decrease of 35% was observed in *C. canadensis* while the others ranged from 7 to 15%. These declines are on par with those reported for essential elements in other plant species and thus, the lack of significance may simply be due to a limited sample number. In one species, *U. alata*, we observed a small, non-significant increase in BSi concentrations under CO_2 enrichment (**Table 1**). We know of only one other study that examined the impact of elevated CO_2 on plant BSi concentrations. In that study of cotton (*Gossypium hirsutum* L.cv. Deltapine 77), the Si concentrations increased by 26%, although the results were also not significant (Huluka et al., 1994).

We observed a decline in BSi concentrations in N enriched trees compared to the control ranging from 8 to 50%, but none were statistically significant (**Table 1**). Similarly, no significant differences between BSi concentration in CO_2 enriched vs. N enriched were found for any of the species. The combined impact of N and CO_2 enrichment again exhibited mixed results. For three species (*A. rubrum, L. styraciflua, U. alata*) we observed BSi declines from 12 to 21% and in one species (*C. canadensis*) we observed an increase of 60% (**Table 1**). Previous work at this same site found no significant differences in leaf N, phosphorus, C, lignin, or total non-structural carbohydrates under elevated CO_2 (Finzi et al., 2001). This is in contrast to other studies that found decreases in N and increases in C concentrations in other tree species (quaking aspen, *Populus tremuloides* and paper birch, *Betula papyrifera*; Lindroth et al., 2001). Elevated CO_2 concentrations also decreased concentrations of N, potassium, phosphorus, and sulfur in Norway spruce (*Picea abies* L. Karst; Roberntz and Linder, 1999). From these and many other studies, foliar chemistry response to elevated CO_2 and N appears to be site-specific. Additionally, although we did not observe statistically significant effects, the trends we document may be ecologically important. For example, Si provides defense against herbivory and the 15% decline we observed in *A. rubrum* could impact feeding preferences of vertebrate and invertebrate consumers. The lack of significance may be in part driven by our sample numbers and thus, a larger study with more data may produce different results.

Silicon uptake by plants is divided into three broad categories (active, passive, or rejective). In active accumulation, plants acquire more Si than they would through water uptake alone. In rejective accumulation, also known as excluder accumulation, Si is taken up at a slower rate than water. Finally, in passive accumulation, water and Si have similar uptake rates (Raven, 1983; Ma et al., 2001b). Plants are assigned one of these categories according to various definitions, a thorough discussion of which is beyond the scope of this paper, but see Carey and Fulweiler (2014) in this Special Issue for a full description. Briefly, previous definitions have been based on Si concentrations in aboveground tissues, on the ratio of Si to calcium, and on the relationship between porewater Si concentration and aboveground tissue Si concentrations (e.g., Jones and Handreck, 1967; Takahashi et al., 1990; Ma et al., 2001a, respectively). More recent work has focused on the presence/absence of Si transporter genes in roots (*Lsi1* and *Lsi2*) and shoots (*Lsi6*) of rice (Ma et al., 2008; Yamaji et al., 2008). Research on accumulation modes in trees is surprisingly lacking. Cornelis et al. (2010b) observed both passive and rejective Si accumulation growth in coniferous tree saplings grown hydroponically. And a decrease in Si concentration with depth was observed in a temperate coniferous forest and designated as active accumulation (Gérard et al., 2008). Adult trees likely rely more heavily on groundwater and thus, in order to precisely determine the mode of Si accumulation, measurements of Si concentrations in groundwater, porewater, and within the tree are needed. Unfortunately, this is beyond the scope of this paper. Therefore, we apply the simplest definition that describes accumulation status as a function of Si concentration in the aboveground tissue alone: active accumulators as >0.46% Si by wt., passive accumulators as between 0.25 and 0.46% Si by wt., and excluders as <0.25% Si by wt. (Ma et al., 2001b; Street-Perrott and Barker, 2008). We acknowledge the limitations of this definition, as aboveground BSi tissue concentrations can be impacted by numerous factors, such as porewater DSi availability and external stressors. However, given our dataset, it is the one most appropriate for us to use. Defining Si accumulation status in trees by linking Si concentrations in aboveground vegetation to changes in porewater and groundwater, and locating Si transporter genes within trees are important areas of future research.

We observed a wide range in foliar BSi concentrations (**Table 1**). The low Si concentrations found in *C. florida* may indicate that these trees excluded H_4SiO_4. Overall however, the BSi concentrations observed in the Duke forest are 40 to 150% higher than those previously reported for similar species (Hodson et al., 2005). One reason for higher concentrations found in the Duke Forest could be the different soil and climate in North Carolina compared to the majority of studies reported in Hodson et al. (2005), which were dominated by northern temperate field sites. Our samples could also have higher BSi concentrations because of the land use legacy at the Duke Face site. In 1983, just 13 years before these experiments started, the forest was cut, trunks were removed, and the remaining material was burned (Finzi et al., 2001). The impact of land use change and disturbance on Si cycling is an emerging topic. From what we currently know, greater DSi losses have been observed following deforestation (Likens et al., 1970; Conley et al., 2008).

The median and mean BSi concentration (0.81 and 0.98 %Si dry wt., respectively) of the species we studied here is higher than many well-known actively Si accumulating groups, including those found in grasses (*Poaceae*) and sedges (*Cyperaceae*; Jones and Handreck, 1967; Raven, 1983; Ma and Takahashi, 2002). Uptake by forest trees has been hypothesized as a mechanism responsible for both the clear seasonal cycle of Si concentrations in stream water (Fulweiler and Nixon, 2005) and the observation of forested watersheds exporting significantly less Si than watersheds dominated by urban-land uses (Carey and Fulweiler, 2012a, 2013b). Given these data, and the known limitations of Si accumulation definitions, we can only hypothesize that this forest is actively accumulating Si. Regardless, the high Si concentrations we observed support the idea that forests are a critical component in regulating the flux of Si from land to the sea.

DUKE FACE SI UPTAKE

The impact of elevated CO_2, N, and combined elevated CO_2 and N led to an approximately 28% increase in net primary production (NPP) at the Duke Face site between 1996 and 2004 (McCarthy et al., 2010). We used the mean NPP over this period to estimate the amount of Si taken up by the Duke forest for the control and elevated CO_2 treatments. We focused on the CO_2 treatment because we do not have BSi concentrations for the *P. taeda* under N fertilization or the combined elevated CO_2 and N fertilization treatments. Additionally, biomass at this site is dominated by *P. taeda*, which comprises ~98% of the tree basal area (Finzi et al., 2001). We calculated foliar Si:C ratios by dividing the treatment specific median %Si value of either *P. taeda* alone or all the species together by 0.47, as C concentrations in biomass are well constrained between 45 and 50%. We then multiplied this Si:C ratio by the known amount of C in each treatment to get a treatment specific Si foliar uptake value (Conley, 2002; Carey and Fulweiler, 2012b). We estimated woody biomass Si uptake using the mean temperate woody biomass %Si of 0.08 (Fulweiler and Nixon, 2005) and again divided by 0.47. We then prorated the amount of Si by the proportion of leaves (30%) versus woody biomass (70%) in a typical forest to determine a total Si uptake rate (**Figure 2**; Litton et al., 2007). Although we observed a decline in Si concentrations under elevated CO_2, the total amount of Si taken up by *P. taeda* was 26% higher at elevated CO_2 where NPP was significantly higher compared to ambient CO_2 (control: 194 kmol Si km^{-2}, elevated CO_2: 251 kmol Si km^{-2}). Together with the hardwood species, Si uptake rate was 20% higher in the elevated compared to ambient CO_2 treatment, although absolute rates of uptake were higher than *P. taeda* alone (elevated $CO_2 = 313$ kmol km^{-2} y^{-1}; ambient $CO_2 = 266$ kmol Si km^{-2}, **Figure 2**). These values are on the high end of those reported for forested systems but well within the reported range. For example, Gérard et al. (2008) reported 157 kmol Si km^{-2} for a Douglas fir forest in France, while Meunier et al. (1999) reported an uptake of over 3400 kmol Si km^{-2} in a bamboo forest.

A review of FACE experiments found that irrespective of ecosystem type, aboveground production increased in the presence of higher CO_2 and trees were more responsive than

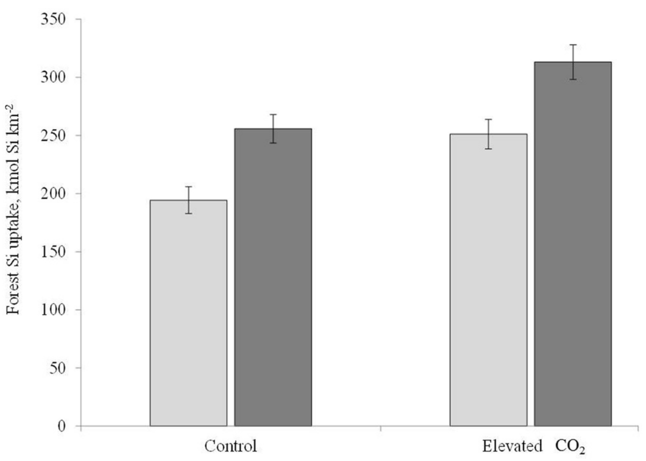

FIGURE 2 | A comparison between the control and elevated CO_2 treatment of forest Si accumulation for the loblolly pine alone (light gray) and for all the species together (dark gray).

herbaceous vegetation (Ward et al., 2013). Other studies have found similar increases in global terrestrial production (Melillo et al., 1993; Nemani et al., 2003). In addition, Pan et al. (2013) found that global forest biomass has increased in established forests. Such changes in primary productivity will also alter Si cycling. In fact, Si accumulating vegetation accounts for 55% of terrestrial NPP (33 Gton C y^{-1}) an amount similar to the C sequestered by marine diatoms (Carey and Fulweiler, 2012b). This terrestrial Si pump has important implications for global climate, as Si cycling helps to control atmospheric CO_2 concentrations through a variety of mechanisms, including chemical weathering of mineral silicates and C occlusion in soil phytoliths (Berner and Berner, 1997; Parr and Sullivan, 2005). In addition, this vegetation also plays a critical role in modulating the amount and timing of Si export from watersheds to downstream receiving waters (Carey and Fulweiler, 2012a, 2013b), which has direct implications for marine C dynamics. Here, we show that anthropogenically driven enhanced NPP may result in an increase in the terrestrial Si pump as forests take up more Si. In turn, the increased terrestrial Si sink may alter Si availability in aquatic systems. Diatoms require N and Si on a one to one molar basis (Redfield, 1963). Thus, the ratio of N to phosphorus to Si (N:P:Si) helps to control the composition and abundance of phytoplankton species assemblages. Human activities, such as fertilizer use and land use change have increased N and P loading to coastal systems worldwide. Phytoplankton respond to these elevated nutrients by increased productivity. At first, diatoms will bloom until all the Si is consumed at which point other non-Si requiring species will flourish (Anderson et al., 2002). The enhanced Si uptake by forests under elevated CO_2 may be another way in which humans are altering nutrient stoichiometry in coastal receiving waters.

Missing from this discussion is the mechanism driving our findings that CO_2 and/or N additions have no significant impact on Si accumulation in aboveground biomass. Of course, with more data and different study sites we might observe a significant impact of these factors on Si accumulation rates in forest or other terrestrial vegetation types. In fact, a recent study found higher Si concentrations in the porewater, sediment, roots, and occasionally the aboveground biomass of a heavily N enriched salt marsh (Carey and Fulweiler, 2013a). Certainly other factors such as changes in water availability and growing season length, as well as warming temperatures, are all factors that might influence foliar BSi accumulation. Here we propose an alternative idea for exploration: we hypothesize that in ecosystems with altered transpiration rates, corresponding changes to leaf Si concentrations will also be observed because Si is delivered to vegetation via water. Higher transpiration rates should, if passive or active accumulation is occurring, result in higher Si concentration in leaves, as Si is typically concentrated at transpiration termini. Conversely, if transpiration rates decline, then less Si will be deposited. Experimental work on Douglas fir saplings found higher Si concentrations that were attributed to greater transpiration in those seedlings (Cornelis et al., 2010b). However, at the Duke FACE experimental site no changes in transpiration were observed and maybe this is why we observed no significant changes in Si concentration (Ward et al., 2013). Complicating these simple ideas is the original hypothesis that motivated this work – enhanced CO_2 lowers elemental composition of leaves such as N, phosphorus, and potassium. Thus, quantifying the interplay between structural changes in vegetation and transpiration rates, as well as water availability under future climate change scenarios, will be a critical next step in our understanding of climate change impacts on terrestrial Si cycling.

CONCLUSION

Our data of BSi concentrations in 6 species from the Duke FACE Experiment does not support our initial hypothesis that elevated CO_2 concentrations would decrease foliar Si content. In fact, we observed no consistent or significant impact of any of the treatments on foliar Si content. However, according to the simplest definition based on aboveground tissue Si concentrations, we did find evidence that four out of the six tree species we studied may be active Si accumulators. These tree species had Si concentrations higher than some of the most well-known Si accumulators (e.g., grasses and sedges). Further, the higher NPP values observed under elevated CO_2 resulted in higher Si uptake rates under elevated CO_2 conditions in the Duke forest. Based on this analysis we hypothesize that anthropogenic change, specifically elevated atmospheric CO_2 concentrations, may increase biological Si pumping in forests, increasing the magnitude of the terrestrial Si pump.

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