Synergism between calcium nitrate applications and fungal endophytes to increase sugar concentration in *Festuca sinensis* under cold stress

Lianyu Zhou[1,2], Chunjie Li[1], James F. White[3] and Richard D. Johnson[4]

[1] State Key Laboratory of Grassland Agro-ecosystems; Key Laboratory of Grassland Livestock Industry Innovation, Ministry of Agriculture and Rural Affairs; Engineering Research Center of Grassland Industry, Ministry of Education; Gansu Tech Innovation Centre, Lanzhou University, Lanzhou, Gansu, China

[2] Key Laboratory of Medicinal Plant and Animal Resources of the Qinghai-Tibetan Plateau, School of Life Science, Qinghai Normal University, Xining, Qinghai, China

[3] Department of Plant Biology, Rutgers University, New Brunswick, NJ, United States of America

[4] Grasslands Research Centre, AgResearch Limited, Palmerston North, New Zealand

ABSTRACT

Epichloë endophytes have been shown to increase tolerance to biotic and abiotic stresses in many cool-season grasses. We investigated the impact of endophyte infection of *Festuca sinensis*, on root metabolic activity, photosynthetic pigments, leaf relative water content (RWC) and soluble carbohydrates in a field experiment carried out during chilling and irrigation with $Ca(NO_3)_2$. A highly significant ($P < 0.001$) correlation for *Epichloë* endophytes was observed for root metabolic activity. $Ca(NO_3)_2$ affected very significantly root metabolic activity and total chlorophyll ($P < 0.001$). Low temperature led to highly significant ($P < 0.001$) reductions in root metabolic activity, RWC, total chlorophyll, chlorophyll a/b ratio, and carotenoid contents. In addition, the fructose concentrations of shoots were greater on the 14th day than on the 28th day and before treatment, whilst the glucose concentration of roots was much higher on the 28th day than before and after 14 days treatment. Moreover, our results indicated that the addition of calcium nitrate contributed to higher levels of total chlorophylls, soluble sugars, sucrose, fructose or glucose in the shoots and roots in both E+ and E- plants during long periods of chilling. These results suggest that *Epichloë* endophyte infection and/or exogenous calcium nitrate can confer better tolerance to cold stress.

Subjects Agricultural Science, Biochemistry, Ecology, Mycology, Plant Science
Keywords *Festuca sinensis*, *Epichloë* endophyte, Cold tolerance, Calcium nitrate, Synergism

INTRODUCTION

Low temperature is one of the main abiotic stresses that retards growth and development of many plants (*Nayyar, Bains & Kumar, 2005*). There is considerable evidence that exposure to low temperature causes a variety of morphological, physiological and biochemical responses, including declines in chlorophyll content (*Hansen, Vogg & Beck, 1996; Lootens, Van Waes & Carlier, 2004*), root metabolic activity (*Sun et al., 2016*), leaf relative water content (RWC) (*Burchett, Niven & Fuller, 2006*), and accumulation of soluble

carbohydrates (*Klotke et al., 2004*; *Stupnikova et al., 2002*). Nitrogen uptake is significantly influenced by low temperature and lack of growth activity of the plant (*Dong et al., 2001*).

Calcium is a critical nutrient that plays a significant role in maintaining the structure and function of the cell wall and cell membrane. Many studies have demonstrated that calcium regulates the processes of plant growth and development, and acts as a second signal to regulate responses of plants to stresses (*Bush, 1995*; *Bowler & Fluhr, 2000*; *Hepler, 2005*), such as drought (*Xu, Li & Zhang, 2013*), temperature (*Monroy, Sarhan & Dhindsa, 1993*; *Tan et al., 2011*; *Yang et al., 2013*; *Karpets et al., 2016*; *Yang et al., 2016*), oxidate (*Schmitz-Eiberger, Haefs & Noga, 2002*), salt (*Navarro, Martínez & Carvajal, 2000*) and metals (*Min et al., 2012*; *Rahman et al., 2016*). Exogenous calcium has been demonstrated to confer protection against cold stress by alleviating chlorophyll and carotenoid degradation (*Schaberg et al., 2011*; *Feng et al., 2010*), increasing antioxidant enzyme activity, increasing root metabolic activity (*Li, Gao & Liu, 2011*; *Feng et al., 2010*; *Liu et al., 2012*), and enhancing sugar contents (*Halman et al., 2008*; *Hawley et al., 2006*). Cold stress promotes endogenous Ca^{2+} level in leaves and roots, moreover, exogenous application of Ca^{2+} increased endogenous Ca^{2+} content under control and cold conditions (*Shi et al., 2014*).

Plant adaptation to stress may be mediated by association with microorganisms (*Rodriguez & Redman, 2008*; *Redman et al., 2011*; *Yin et al., 2014*). *Epichloë* endophytes (formerly *Neotyphodium* spp.; *Leuchtmann et al., 2014*) have been found in many cool-season grasses and can improve the ability of host grasses to withstand biotic and abiotic stresses (*Johnson et al., 2013*; *Yin et al., 2014*; *Xia et al., 2016*; *Ma, Christensen & Nan, 2015*; *Song et al., 2015*). For example, grass infection with *Epichloë* endophytes improves photosynthetic pigment abundance (*Rozpadek et al., 2015*), and confers benefits to grasses in response to cold stress including increased root biomass and increased fungal secondary metabolite production (*Latch, Hunt & Musgrave, 1985*; *Zhou et al., 2015a*; *Chen et al., 2016*). In addition, other symbioses are reported to affect photosynthetic pigment degradation, leaf relative water content, soluble sugars and other physiological markers for chilling stress (*Mishra et al., 2011*; *Zhou et al., 2012*; *Ding et al., 2011*; *Wang et al., 2016*). In such instances, exogenous chemical applications or symbiotic technology serve to improve plant adaptation to low temperature stress (*Halman et al., 2008*; *Yang et al., 2016*; *Jeon et al., 2010*; *Zhou & Guo, 2009*; *Redman et al., 2011*). The combined application of microorganism and plant growth regulator has been found to protect plant from low temperature stress (*Zhou et al., 2012*). However, information about the influence of exogenous calcium nitrate and *Epichloë* on physiological response of plant adaptation to cold stress is rather scanty.

Festuca sinensis is an important cool-season grass species grazed by cattle and sheep, especially in cool and semi-arid regions of China (*Nan & Li, 2000*). This grass species is frequently host to an asexual symptomless *Epichloë* sp. that resides in the apoplastic spaces of the leaf sheaths, culms and seeds (*Peng et al., 2013*). Several reports have indicated that *Epichloë* endophytes improve the response of *F. sinensis* to stresses such as drought, pathogens and cold under controlled conditions (*Peng et al., 2013*; *Zhou et al., 2015b*). Cold winter conditions can lead to damage or death of grasses. However, despite this survival of

F. sinensis it is still stressed under long-term cold exposure in grasslands of northwestern China.

A previous study has suggested that cold stress negatively affects nitrogen uptake by plants (*Dong et al., 2001*). The aim of the present study was to examine the role of exogenously applied calcium nitrate and *Epichloë* endophytes in stimulation of root metabolic activity, photosynthetic pigment accumulation, RWC and soluble carbohydrates, and how these may improve *F. sinensis* survival under cold conditions in the field.

MATERIALS & METHODS

Growth

Festuca sinensis seeds (E+) that were naturally infected with the endophyte were collected in September 2012 from Xiahe county, Gansu Provence in China (3,000 m, 98°23′E, 34°23′N). To obtain endophyte free (E-) seeds, *Epichloë* endophyte infected seeds (E+) were treated with 300 times dilution of fungicide thiophanate-methyl for 8 h to kill the fungus (*Yao et al., 2013*). The infection status of each plant was determined via microscopic (Olympus, Japan) examination of aniline blue-stained leaf sheath pieces. Vegetatively-propagated E+ and E- plants, grown in a constant temperature greenhouse (soil:nutritive medium, 1:1, v/v, 22 °C, 16 h light/8 h dark) as previously described (*Zhou et al., 2015a*) were used in subsequent experiments. The clones consisting of one tiller were planted during May 2014 in the experimental field of the College of Pastoral Agriculture Science and Technology, Yuzhong campus of Lanzhou University (loess soil, 104°39′E, 35°89′N, Altitude 1,653 m), and were watered as required. Before the onset of experimental treatments, the soil was tilled to reduce differences in soil fertility. The trials were arranged using a split-split-plot design with a total of 216 plants of *F. sinensis*, where three $Ca(NO_3)_2$ concentrations were randomly assigned into three replicates as main plot treatments, two endophyte statuses (i.e., E+ and E-) were randomly assigned into the main plots as split plot treatments, and randomly selected plants were sampled at three times in two strips (two plants each time) as the split-split plots in two strips (Fig. S1). There were 18 treatments (2 endophyte types ×3 $Ca(NO_3)_2$ concentrations ×3 sampling times), with three repetitions for each treatment and 12 plants for each repetition (i.e., 36 plants for each treatment). There was a distance of 22 cm between strips (i.e., endophyte types) and 18 cm between plants within a strip. For split plot, plot size was 20 m² with two surrounding-protection strips and two sampling strips. The strips of plants were 0.5 m apart from each other. Plants were grown for 5 months under natural conditions. After this period, plants were irrigated from 29 October to 28 November once a week for 4 weeks with 15 mL $Ca(NO_3)_2$ solution at three concentrations, 0 (CK), 25 and 50 mM, respectively. The herbage and roots of 6 different plants under each $Ca(NO_3)_2$ solution condition for either E+ or E- plants were randomly harvested from three repetitions in the morning on day 0 (before plant treatment), 14 and 28, respectively. Daily air temperature ranged from 3 °C to 14 °C during the day and −7 ° C to 2 °C during the night during the experiment according to the data from http://www.tianqihoubao.com/lishi/yuzhong.html (Fig. 1).
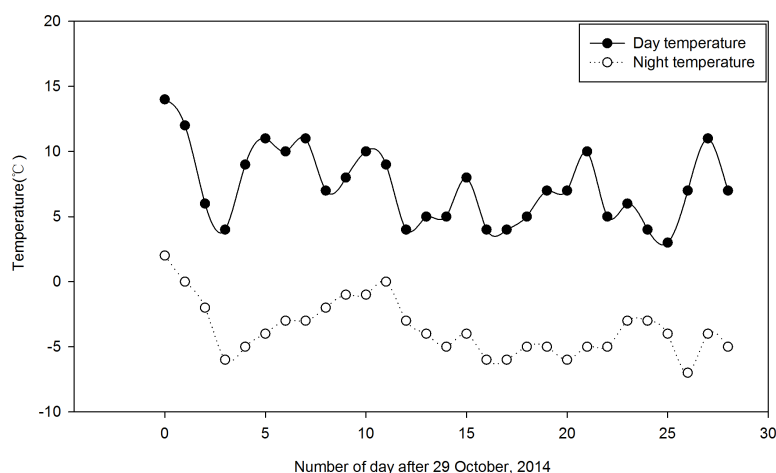
Figure 1   Daily air temperature profile of the field during the study period (29 October to 28 November, 2014). Each data point indicates the average temperature.

Full-size 🖼 DOI: 10.7717/peerj.10568/fig-1

Determination of root metabolic activity

Root metabolic activity was measured using the modified *2, 3, 5-* triphenyltetrazolium chloride (TTC) method (*Li, 2000*). This method provides an estimate of metabolic activity in roots by reflecting aerobic respiration rates (*Li, 2000*). To conduct this assay, root tips (0.5 g fresh weigh, FW) were dipped in a solution containing five mL of 0.4% TTC and five mL of 1/15 M phosphate buffer solution (pH 7.0), and incubated at 37 °C for 1 h in the dark. Then, 2 mL of 1 M H_2SO_4 was added to the mixture to stop the reaction. The root was removed carefully, wiped and then ground with a small amount of ethyl acetate and quartz sand in a mortar to extract triphenyl formazan (TTF). The residue was washed 2–3 times with a small amount of ethyl acetate and added to the first extraction and made up to a volume of 10 mL. Root metabolic activity was measured using a spectrophotometer (SP-723) with an absorbance of 485 nm and using H_2SO_4 as a blank.

Analysis of chlorophyll and carotenoid contents

Chlorophyll and carotenoid contents were measured according to the modified method of *Li (2000)*. Leaves (0.05 g FW) were cut and extracted with 80% acetone for 48 h in the dark. Absorbance was quantified at 663, 645 and 470 nm using a spectrophotometer. Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents were calculated according to the following equations: chlorophyll a (C_a) = (12. 72 × A_{663}) (2.59 × A_{645}), chlorophyll b (C_b) = (22.88 × A_{645}) (4.86 × A_{663}), total chlorophyll $(C_a + C_b)$ = (8.02 × A_{663})+ (20.20 × A_{645}), carotenoid = [(1,000 × A_{663}) (3.27 × C_a) (104× C_b)]/229.

Assessing leaf relative water content

Leaves were cut from the plant, weighed immediately (fresh weigh, FW), floated in water for 24 h to achieve turgidity (saturated weight, SW), then oven-dried at 70 °C and weighed again (dry weight, DW). RWC of leaves was calculated according to the formula: [(FW DW)/(SW DW)] ×100%.

Soluble sugar, sucrose, fructose and glucose analysis

Soluble sugars, sucrose, fructose and glucose were determined using the methods of *Shanghai Institute of Plant Physiology (1999)*. Powdered dry samples (0.05 g) were extracted with four mL of 80% ethanol in a water bath at 80 °C for 30 min. The residue was re-extracted twice, and the three extracts were combined. The supernatants were used to determine the soluble sugars, sucrose, fructose and glucose concentrations.

For determination of soluble sugars, one mL of the filtered solution was mixed with 0.5 mL anthrone solution (1 g anthrone dissolved in 50 mL ethyl acetate) and five mL H_2SO_4 and heated at 90 °C for 10 min. The mixture was immediately placed in tap water to cool and the absorbance measured at 620 nm. The concentrations of total soluble sugar were determined using glucose as the standard.

For determination of sucrose, 0.1 mL of the filtered solution was mixed with 50 μL of 2 M NaOH and incubated at 100 °C for 5 min, after which it was immediately cooled. The mixture was added to 0.7 mL of 30% HCl and 0.2 mL of 0.1% resorcinol, heated in a water bath at 80 °C for 10 min and after cooling absorbance was determined at 480 nm. The sucrose concentration was calculated using sucrose as the standard.

For determination of fructose, 0.1 mL of the filtered solution was mixed well with 0.2 mL of 0.1% resorcinol and 0.7 mL H_2O, heated in a water bath at 80 °C for 10 min and cooled. Absorbance at 480 nm was determined. The fructose concentration was measured using fructose as the standard.

For analysis of glucose concentration, four mL of enzyme solution including 1 mg o-dianisidine dihydrochloride, 0.1 mg horseradish peroxidase (CAS:9003-99-0, Solarbio) and 1 μL glycose oxidase (1,000 U/mL, dissolved in 0.1 M acetate buffer, pH 5.5, CAS:9001-37-0, Solarbio) was placed at 30 °C until equilibrium after which two mL of the extracted sugar solution was added and mixed well for 5 min at 30 °C. The reaction was stopped by addition of eight mL of 10 M H_2SO_4, and the absorbance was determined at 460 nm. The glucose concentration was determined using glucose as the standard.

Statistical analysis

Statistical analyses were performed with DPS software, Version 9.50. Data were presented as the mean ± SE and means were compared by Fishers Least Significant Differences (LSD) test at 0.05 probability level. Homoscedasticity was evaluated by Levene's test after some data were logarithmic, sine or cosine transformed. Correlation analysis was conducted between indicators measured and temperature using Spearman's rho method (SPSS 16).

RESULTS

Root metabolic activity

Highly significant ($P < 0.001$) effects of *Epichloë* endophyte, calcium nitrate treatment, or treatment time were found on root metabolic activity of *F. sinensis*. In addition, there were some interactions between *Epichloë* endophyte, calcium nitrate treatment, and treatment time for root metabolic activity ($P < 0.001$, Table 1). As shown in Table 2 and Fig. 2, the E+ plants had higher root metabolic activity compared to the E- plants before

Table 1 Results of split-split-plot ANOVA for the effects of *Epichloë* endophyte (E), calcium nitrate treatment, and treatment time (T) on root metabolic activity, leaf relative water content, chlorophyll and carotenoid contents of *F. sinensis* under cold field conditions.

Level	Source	df	Root metabolic activity		Relative water content of leaf		Total chlorophyll		Chlorophyll a/b ratio		Carotenoid	
			F	p	F	p	F	p	F	p	F	p
Whole plot	Ca(NO₃)₂	2	460.247	0.000	10.425	0.026	254.385	0.000	12.557	0.019	18.098	0.010
Split plot	E	1	168.300	0.000	13.744	0.010	0.046	0.838	2.779	0.147	0.042	0.845
	Ca(NO₃)₂ ×E	2	40.292	0.000	5.565	0.043	11.682	0.009	2.099	0.204	10.575	0.011
	T	2	1047.777	0.000	373.569	0.000	857.792	0.000	141.541	0.000	32.000	0.000
Split-split plot	Ca(NO₃)₂ ×T	4	60.018	0.000	5.572	0.003	120.014	0.000	1.651	0.194	29.495	0.000
	E ×T	2	16.922	0.000	58.827	0.000	31.830	0.000	4.687	0.019	15.141	0.000
	Ca(NO₃)₂ ×E ×T	4	14.402	0.000	3.946	0.013	37.855	0.000	1.574	0.214	9.478	0.000

Table 2 Results of one-way ANOVA for the effects of treatment time or calcium nitrate treatment on root metabolic activity, leaf relative water content, chlorophyll and carotenoid contents of *F. sinensis* under cold field conditions.

Treatment	df	Root metabolic activity		Relative water content of leaf		Total chlorophyll		Chlorophyll a/ b ratio		Carotenoid	
		F	p	F	p	F	p	F	p	F	p
Time	0 d	5	30.759	0.000	0.861	0.534	32.396	0.000	1.239	0.350	1.198	0.367
	14 d	5	22.468	0.000	8.025	0.002	10.784	0.000	1.114	0.403	4.535	0.015
	28 d	5	169.141	0.000	15.491	0.000	28.544	0.000	1.126	0.398	8.233	0.001
Ca(NO$_3$)$_2$	0 mM	5	64.093	0.000	47.761	0.000	20.323	0.000	6.549	0.004	16.858	0.000
	25 mM	5	108.670	0.000	1.317	0.321	116.833	0.000	2.802	0.067	13.073	0.000
	50 mM	5	250.528	0.000	52.350	0.000	17.645	0.000	10.407	0.000	4.991	0.011

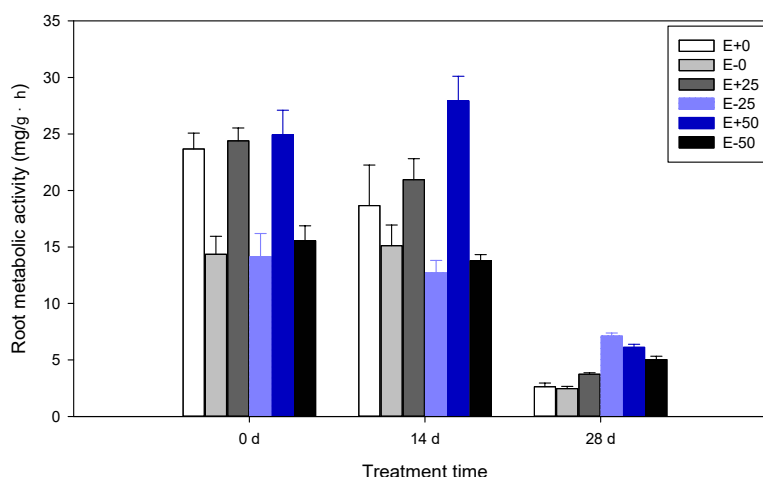**Figure 2 Root metabolic activity of *F. sinensis* with and without *Epichloë* endophyte under different Ca(NO$_3$)$_2$ treatments during the study period.** Data represent means ± standard error (SE). Means within each graph followed by different lower case letters differ statistically among a given treatment time ($P < 0.05$). Means within each graph followed by different upper case letters differ statistically under the same calcium nitrate treatment ($P < 0.05$). E+0, E+25, and E+50 represent endophyte-infected (E+) plants irrigated with Ca(NO$_3$)$_2$ solution at the three concentrations 0, 25 or 50 mM, respectively. E-0, E-25, and E-50 represent non-infected (E–) plants irrigated with Ca(NO$_3$)$_2$ solution at the three concentrations 0, 25 or 50 mM, respectively.

Full-size 🖼 DOI: 10.7717/peerj.10568/fig-2

treatment with calcium nitrate ($P < 0.05$). On the 14th day, the presence of endophyte increased root metabolic activity comparing the E+ vs. E- plants, and E+ plants with 50 mM Ca(NO$_3$)$_2$ treatment [50Ca(NO$_3$)$_2$] had the highest level of root metabolic activity among all treatments. On the 28th day root metabolic activities of E- with 25 mM Ca(NO$_3$)$_2$ treatment [25Ca(NO$_3$)$_2$] were significantly higher than others ($P < 0.05$), and significant differences in E+ or E- plants were also observed ($P < 0.05$). When analyzing time shifts in root metabolic activities, samples except for E+ plants with 50Ca(NO$_3$)$_2$ had significantly higher root metabolic activity on the 14th day than on the 28th day, and lower on the 14th day than before treatment ($P < 0.05$).

Chlorophyll and carotenoid contents

Highly significant ($P < 0.001$) effects of calcium nitrate treatment and treatment time were detected on total chlorophyll of *F. sinensis*. In addition, there were some interactions between *Epichloë* endophyte or calcium nitrate treatment, and treatment time for total chlorophyll ($P < 0.001$, Table 1). There were significant ($P < 0.05$) effects of *Epichloë* status on chlorophyll contents before treatment with calcium nitrate (Table 3). Similarly, on the 14th day, the chlorophyll contents of E+ plants with 25Ca(NO$_3$)$_2$ were significantly ($P < 0.05$) higher than either E- plants or E+ plants with 50Ca(NO$_3$)$_2$. Furthermore, calcium nitrate addition significantly enhanced the chlorophyll contents in E- plants. On the 28th day the chlorophyll contents of E- plants with 25Ca(NO$_3$)$_2$ were the highest among all plants, and the chlorophyll contents of E+ plants supplied with 50Ca(NO$_3$)$_2$ were significantly higher than the other E+ plants ($P < 0.05$). In addition, there was time effect

Table 3 Chlorophyll and carotenoid contents of *F. sinensis* with and without *Epichloë* endophyte under two different Ca(NO₃)₂ treatments during the study period.

Day (d)	Calcium nitrate treatment (mM)	Plant	Total chlorophyll (mg/g)	Chlorophyll a/b ratio	Carotenoid (mg/g)
0	0	E+	1.85 ± 0.05^{aA}	3.47 ± 0.08^{aBC}	0.0758 ± 0.0094^{aB}
		E–	1.55 ± 0.06^{bB}	3.51 ± 0.05^{aBC}	0.0873 ± 0.0091^{aA}
	25	E+	1.88 ± 0.07^{aB}	3.46 ± 0.11^{aA}	0.0836 ± 0.0077^{aB}
		E–	1.56 ± 0.01^{bB}	3.50 ± 0.17^{aA}	0.0779 ± 0.0157^{aB}
	50	E+	1.93 ± 0.05^{aA}	3.47 ± 0.12^{aA}	0.0916 ± 0.0088^{aA}
		E–	1.62 ± 0.05^{bB}	3.64 ± 0.03^{aA}	0.0762 ± 0.0089^{aB}
14	0	E+	1.45 ± 0.15^{cBC}	3.64 ± 0.19^{aAB}	0.0833 ± 0.0113^{aB}
		E–	1.47 ± 0.08^{cBC}	3.86 ± 0.16^{aA}	0.1117 ± 0.0161^{abA}
	25	E+	2.08 ± 0.10^{aA}	3.77 ± 0.06^{aA}	0.1408 ± 0.0087^{aA}
		E–	1.81 ± 0.06^{abA}	3.76 ± 0.21^{aA}	0.1246 ± 0.0174^{abA}
	50	E+	1.69 ± 0.15^{bcA}	3.64 ± 0.15^{aA}	0.1003 ± 0.0282^{bcA}
		E–	1.76 ± 0.15^{bA}	3.82 ± 0.07^{aA}	0.1258 ± 0.0103^{abA}
28	0	E+	0.86 ± 0.12^{cCD}	2.95 ± 0.42^{aC}	0.1460 ± 0.0092^{aA}
		E–	0.76 ± 0.03^{cD}	2.78 ± 0.33^{aC}	0.0746 ± 0.0132^{bB}
	25	E+	0.74 ± 0.07^{cC}	2.73 ± 0.44^{aA}	0.0659 ± 0.0111^{bC}
		E–	1.64 ± 0.10^{aB}	3.15 ± 0.16^{aA}	0.1374 ± 0.0262^{aA}
	50	E+	1.10 ± 0.16^{bB}	3.08 ± 0.23^{aB}	0.0517 ± 0.0184^{bB}
		E–	1.27 ± 0.16^{bB}	3.09 ± 0.20^{aB}	0.1487 ± 0.0541^{aA}

Notes.
E+ and E- represent endophyte-infected and non-infected plants, respectively. Data represent means ± standard error (SE). Means followed by different lowercase letters differ statistically among a given treatment time ($P < 0.05$). Means followed by different uppercase letters differ statistically under the same calcium nitrate treatment ($P < 0.05$).

on chlorophyll contents (Table 2). Chlorophyll contents in both control and treated-plants showed significant changes throughout the experiment (Table 3, $P < 0.05$). The chlorophyll contents for the control and 50Ca(NO₃)₂-treated E- plants, and 50Ca(NO₃)₂-treated E+ plants were much lower on the 28th day than before and after 14 days treatment ($P < 0.05$), however the chlorophyll contents in 25Ca(NO₃)₂-treated E- plants were greater on the 14th day than on the 28th day and before treatment ($P < 0.05$).

Treatment time was the only highly significant ($P < 0.001$) effect detected on chlorophyll a/b ratio for *F. sinensis* (Table 1). Apart from that, not all of the chlorophyll a/b ratios in plants on the 28th day were much lower than on the 14th day and before treatment (Tables 2 and 3, $P < 0.05$).

Highly significant effect of treatment time was detected for carotenoids for *F. sinensis*. In addition, there were some interactions between *Epichloë* endophyte, calcium nitrate treatment, and treatment time for carotenoid content ($P < 0.001$, Table 1). On the 14th day carotenoid contents of E+ plants with 25Ca(NO₃)₂ were significantly greater than those of untreated E+ plants ($P < 0.05$). Similarly on the 28th day, carotenoid contents of the control E+ plants were much greater than those of treated E+ plants and control E- plants, moreover calcium nitrate addition significantly increased carotenoid contents of the E- plants ($P < 0.05$). A significant time effect on carotenoid content was also observed ($P < 0.05$). Carotenoid contents of treated E- plants before treatment were much lower
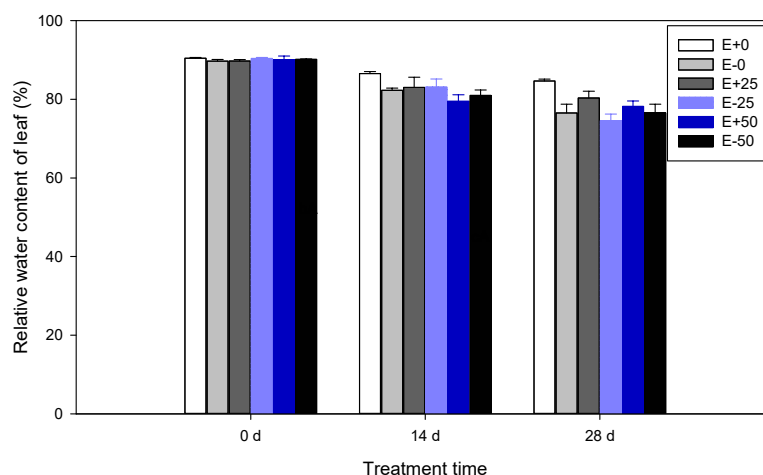
Figure 3 Relative water content of leaf in *F. sinensis* with and without *Epichloë* endophyte under different Ca(NO₃)₂ treatments during the study period. Data represent means $\pm$ standard error (SE). Means within each graph followed by different lower case letters differ statistically among a given treatment time ($P < 0.05$). Means within each graph followed by different upper case letters differ statistically under the same calcium nitrate treatment ($P < 0.05$). E+0, E+25, and E+50 represent endophyte-infected (E+) plants irrigated with Ca(NO₃)₂ solution at the three concentrations 0, 25 or 50 mM, respectively. E-0, E-25, and E-50 represent non-infected (E–) plants irrigated with Ca(NO₃)₂ solution at the three concentrations 0, 25 or 50 mM, respectively.

Full-size 🖼 DOI: 10.7717/peerj.10568/fig-3

than on the 14th day and 28th day ($P < 0.05$), control E- and E+ with 50Ca(NO₃)₂ for carotenoid contents were significantly ($P < 0.05$) decreased on the 28th day compared to on the day zero and the 14th day, and the carotenoid contents in control E+ sharply increased on the 28th day.

Leaf relative water content (RWC)

A highly significant ($P < 0.001$) effect of treatment time was detected for relative water content of *F. sinensis*. In addition, there was significant *Epichloë* endophyte-by-treatment time interactions for RWC ($P < 0.001$, Table 1). Relative water contents of leaves were observed to decline significantly ($P < 0.05$) as time passed after treatment (Table 2, Fig. 3). There were marked water increases in control E+ compared with control E- leaves on the 14th day and 28th day ($P < 0.05$). On the 14th day RWC of E+ plants with 50Ca(NO₃)₂ were significantly ($P < 0.05$) less than those of the control E+ plants. In addition, on the 28th day RWC of E+ plants with 0 and 25Ca(NO₃)₂ were maintained at a significantly ($P < 0.05$) higher level than those of other plants.

Soluble carbohydrates

Highly significant ($P < 0.001$) effects of treatment time or *Epichloë* endophyte were detected for soluble sugars in the shoots or roots of *F. sinensis*. In addition, there were some interactions between *Epichloë* endophyte, calcium nitrate treatment, and treatment time for soluble sugars in the shoots ($P < 0.001$, Table 4). Before calcium nitrate treatment soluble sugars in the shoots were not significantly different across all treatments, whereas soluble sugars in the roots were greater in E+ plants compared with E- plants ($P < 0.05$,

Zhou et al. (2021), *PeerJ*, DOI 10.7717/peerj.10568

11/25

Table 4 Results of split-split-plot ANOVA for the effects of *Epichloë* endophyte (E), calcium nitrate treatment, and treatment time (T) on soluble carbohydrates of shoot and root in *F. sinensis* under cold field conditions.

Level	Source	df	Soluble sugar concentration				Sucrose concentration				Fructose concentration				Glucose concentration			
			Shoot		Root		Shoot		Root		Shoot		Root		Shoot		Root	
			F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Whole plot	$Ca(NO_3)_2$	2	2.418	0.205	1.801	0.277	0.656	0.567	3.150	0.151	2.621	0.187	0.632	0.577	2.096	0.238	2.435	0.203
Split plot	E	1	87.410	0.000	2.740	0.149	0.062	0.812	0.904	0.378	0.724	0.428	7.624	0.033	1.369	0.286	0.008	0.934
	$Ca(NO_3)_2 \times E$	2	10.056	0.012	2.311	0.180	11.669	0.009	8.116	0.020	1.389	0.319	9.016	0.016	4.902	0.055	0.102	0.905
	T	2	563.155	0.000	995.675	0.000	33.611	0.000	1716.937	0.000	68.166	0.000	47.395	0.000	59.602	0.000	104.878	0.000
Split-split plot	$Ca(NO_3)_2 \times T$	4	4.875	0.005	1.553	0.219	0.356	0.837	3.489	0.022	2.094	0.113	3.550	0.021	1.659	0.192	2.631	0.059
	$E \times T$	2	47.415	0.000	9.319	0.001	0.494	0.616	2.482	0.105	0.484	0.622	10.790	0.0005	1.745	0.195	0.006	0.995
	$Ca(NO_3)_2 \times E \times T$	4	16.478	0.000	1.151	0.357	3.654	0.018	5.023	0.004	1.821	0.158	4.481	0.008	4.582	0.007	0.097	0.982

Tables 5–7). On the 14th day E+ plants with 25Ca(NO$_3$)$_2$ significantly accumulated soluble sugars in the shoots ($P <$ 0.05) compared to E- plants. On the 28th day the E+ plants had significantly higher soluble sugar concentrations in the shoots than the E- plants under control and 25Ca(NO$_3$)$_2$ ($P <$ 0.05). However, calcium nitrate application greatly elevated soluble sugars in the roots in E- plants. In addition, there were substantial time changes in soluble sugar concentrations (Tables 6 and 7). The soluble sugar concentrations of E- plants, control and 50Ca(NO$_3$)$_2$ treated E+ plants were much higher on the 28th day than before and after 14 days treatment ($P <$ 0.05). Soluble sugar concentrations for 25Ca(NO$_3$)$_2$ treated E+ plants peaked on the 14th day ($P <$ 0.05).

The highly significant ($P <$ 0.001) effects of treatment time were detected for sucrose levels of shoots and roots of *F. sinensis*. In addition, there were some significant interactions between *Epichloë* endophyte, calcium nitrate treatment, or treatment time for sucrose levels of shoots or roots ($P <$ 0.05, Table 4). The sucrose levels of shoots and roots presented in Tables 6 and 7. After 14 d of calcium nitrate treatment sucrose concentrations of E+ shoots were significantly enhanced by 25Ca(NO$_3$)$_2$ treatment when compared with control E- shoots ($P <$ 0.05). On the 28th day sucrose concentrations of E+ shoots with 25Ca(NO$_3$)$_2$ were significantly greater than those of the control or 25Ca(NO$_3$)$_2$ treated E- plants ($P <$ 0.05). In addition, on the 28th day control E+ roots accumulated higher sucrose concentrations compared to those of other plants, and E+ with 25Ca(NO$_3$)$_2$ increased more rapidly compared to those of E+ with 50Ca(NO$_3$)$_2$ ($P <$ 0.05). With respect to the time series, the sucrose concentrations of roots were higher on the 28th day than on the 14th day and before treatment ($P <$ 0.05).

The highly significant ($P <$ 0.001) effects of treatment time were detected for fructose concentrations of shoots and roots of *F. sinensis*. In addition, there were some interactions between *Epichloë* endophyte and treatment time for fructose concentrations of roots ($P <$ 0.001, Table 4). On the 14th day exogenous calcium nitrate significantly increased fructose concentrations of roots in E- plants ($P <$ 0.05, Tables 5–7). Fructose concentrations of plants shoots were significantly different across the time points (before treatment, on the 14th and 28th day). Fructose concentrations of E+ shoots treated were significantly higher on the 14th day than before treatment and on the 28th day, control E- before treatment were lower than on the 14th day and 28th day ($P <$ 0.05). The fructose concentrations of roots was significantly less on the 14th day than before treatment or on the 28th day ($P <$ 0.05).

The highly significant ($P <$ 0.001) effects of treatment time were detected on the glucose concentrations of shoots and roots of *F. sinensis* (Table 4). On the 28th day glucose concentrations of control E+ shoots were significantly higher than those of control E- and E+ with 25Ca(NO$_3$)$_2$ ($P <$ 0.05, Table 6). Regarding given treatment time point variance, glucose concentrations of both E- roots supplied with Ca(NO$_3$)$_2$ and control E+ were higher on the 28th day than on the 14th day and before treatment ($P <$ 0.05), while other roots in glucose concentrations on the 14th day were much more than before treatment, and lower than on the 28th day ($P <$ 0.05).

Table 5   Results of one-way ANOVA for the effects of treatment time or calcium nitrate treatment on soluble carbohydrates of shoot and root in *F. sinensis* under cold field conditions.

Treatment		df	Soluble sugar concentration				Sucrose concentration				Fructose concentration				Glucose concentration			
			Shoot		Root		Shoot		Root		Shoot		Root		Shoot		Root	
			F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Time	0 d	5	0.623	0.686	2.824	0.065	0.873	0.527	1.148	0.388	0.774	0.587	0.696	0.637	0.201	0.956	1.199	0.366
	14 d	5	35.378	0.000	1.191	0.370	1.438	0.280	0.445	0.809	0.370	0.859	16.387	0.000	2.005	0.150	0.052	0.998
	28 d	5	15.033	0.000	4.331	0.017	2.740	0.071	6.057	0.005	0.071	0.996	1.368	0.303	3.991	0.023	1.857	0.176
Ca(NO$_3$)$_2$	0 mM	5	45.897	0.000	114.614	0.000	12.323	0.000	581.967	0.000	11.744	0.000	27.265	0.000	20.471	0.000	35.751	0.000
	25 mM	5	107.412	0.000	94.298	0.000	7.913	0.002	106.278	0.000	16.352	0.000	7.122	0.003	13.305	0.000	11.899	0.000
	50 mM	5	173.728	0.000	181.708	0.000	1.349	0.309	558.889	0.000	7.392	0.002	5.099	0.010	5.852	0.006	7.043	0.003

Table 6 Soluble carbohydrates in shoot of *F. sinensis* with and without *Epichloë* endophyte under different Ca(NO$_3$)$_2$ treatments during the study period.

Day (d)	Calcium nitrate (mM)	Plant	Soluble sugar concentration (mg/g)	Sucrose concentration (mg/g)	Fructose concentration (mg/g)	Glucose concentration (mg/g)
0	0	E+	13.90 ± 0.41[aB]	2.14 ± 0.05[aB]	9.46 ± 0.84[aC]	1.70 ± 0.20[aC]
		E−	14.22 ± 0.27[aB]	2.14 ± 0.08[aA]	8.57 ± 0.33[aC]	1.73 ± 0.08[aA]
	25	E+	13.90 ± 0.47[aC]	2.16 ± 0.08[aB]	8.90 ± 0.67[aB]	1.66 ± 0.13[aA]
		E−	14.30 ± 0.37[aB]	2.14 ± 0.03[aA]	8.68 ± 0.69[aC]	1.73 ± 0.10[aC]
	50	E+	13.85 ± 0.40[aB]	2.17 ± 0.06[aA]	9.46 ± 0.19[aC]	1.66 ± 0.25[aA]
		E−	14.04 ± 0.52[aB]	2.23 ± 0.08[aA]	9.01 ± 1.07[aC]	1.76 ± 0.07[aC]
14	0	E+	13.39 ± 0.48[bB]	2.20 ± 0.07[abB]	10.73 ± 0.24[aAB]	1.33 ± 0.10[aB]
		E−	13.00 ± 0.94[bB]	2.13 ± 0.12[bA]	12.34 ± 1.54[aA]	1.38 ± 0.10[aB]
	25	E+	15.52 ± 0.52[aB]	2.32 ± 0.08[aB]	14.01 ± 0.84[aA]	1.58 ± 0.06[aA]
		E−	13.20 ± 0.10[bB]	2.17 ± 0.14[abA]	12.79 ± 0.96[aA]	1.30 ± 0.18[aB]
	50	E+	13.92 ± 0.18[bB]	2.28 ± 0.02[abA]	13.90 ± 2.08[aA]	1.49 ± 0.23[aA]
		E−	13.37 ± 0.29[bB]	2.18 ± 0.14[abA]	12.23 ± 0.47[aAB]	1.31 ± 0.08[aB]
28	0	E+	18.09 ± 0.28[aA]	2.69 ± 0.16[abA]	10.34 ± 0.51[aB]	2.34 ± 0.06[aA]
		E−	16.14 ± 0.25[cA]	2.33 ± 0.13[bcA]	10.57 ± 0.58[aB]	1.87 ± 0.23[bA]
	25	E+	18.53 ± 0.52[aA]	2.69 ± 0.20[aA]	10.34 ± 1.17[aB]	1.72 ± 0.03[bA]
		E−	17.18 ± 0.58[bA]	2.25 ± 0.15[cA]	10.68 ± 1.02[aB]	2.00 ± 0.09[abA]
	50	E+	18.16 ± 0.19[aA]	2.48 ± 0.32[abcA]	10.68 ± 1.07[aBC]	1.96 ± 0.36[abA]
		E−	18.04 ± 0.39[aA]	2.64 ± 0.17[abA]	10.46 ± 1.35[aBC]	2.12 ± 0.11[abA]

Notes.

E+ and E- represent endophyte-infected and non-infected plants, respectively. Data represent means ± standard error (SE). Means followed by different lowercase letters differ statistically among a given treatment time ($P < 0.05$). Means followed by different uppercase letters differ statistically under the same calcium nitrate treatment ($P < 0.05$).

Correlation of temperature and indicators measured of *F. sinensis*

Under field conditions, temperature and carotenoid concentration, shoot and root fructose concentrations, or glucose concentration of shoot were poorly correlated (Table 8). There were significantly positive correlation between total chlorophyll or chlorophyll a/b ratio and temperature ($P < 0.05$). The correlativity between temperature and root activity, and relative water content of leaf were significantly higher at the 0.01 level, the correlativity coefficients were 0.734 and 0.761, respectively. However soluble sugar concentration of shoot and sucrose concentration of root negatively correlated with temperature ($P < 0.05$), and soluble sugar concentration of root, sucrose concentration of shoot and glucose concentration of root negatively correlated with temperature ($P < 0.01$) with the Spearman correlations being 0.944, 0.682, and 0.866, respectively.

DISCUSSION

Low temperature induces phenotypic, physiological and biochemical changes in plants. Low temperature or growth inactivity of the plant may reduce uptake of nitrogen (NH$_4$NO$_3$). In this study we compared the changes in physiological performance and soluble sugar concentrations in 5-month-old *F. sinensis* E+ and E- plants when supplied with exogenous Ca(NO$_3$)$_2$ to elucidate the mechanism of plant survival during cold winter conditions.

Table 7 Soluble carbohydrates in root of *F. sinensis* with and without *Epichloë* endophyte under different Ca(NO₃)₂ treatments during the study period.

Day (d)	Calcium nitrate (mM)	Plant	Soluble sugar concentration (mg/g)	Sucrose concentration (mg/g)	Fructose concentration (mg/g)	Glucose concentration (mg/g)
0	0	E+	11.29 ± 0.21^{aC}	1.73 ± 0.01^{aB}	6.23 ± 0.47^{aAB}	0.85 ± 0.03^{aC}
		E−	10.40 ± 0.27^{bC}	1.75 ± 0.06^{aB}	5.57 ± 0.67^{aABC}	0.81 ± 0.01^{aC}
	25	E+	11.24 ± 0.32^{aC}	1.74 ± 0.08^{aB}	5.57 ± 0.67^{aB}	0.82 ± 0.04^{aC}
		E−	10.42 ± 0.85^{abC}	1.78 ± 0.06^{aB}	5.79 ± 0.69^{aAB}	0.84 ± 0.02^{aB}
	50	E+	11.25 ± 0.27^{aC}	1.70 ± 0.03^{aB}	5.68 ± 0.51^{aA}	0.81 ± 0.05^{aC}
		E−	10.37 ± 0.64^{bC}	1.78 ± 0.03^{aB}	6.23 ± 0.88^{aA}	0.86 ± 0.02^{aB}
14	0	E+	14.65 ± 0.18^{aB}	1.73 ± 0.07^{aB}	4.68 ± 0.19^{abC}	1.05 ± 0.13^{aB}
		E−	14.65 ± 0.80^{aB}	1.75 ± 0.06^{aB}	2.34 ± 0.51^{cD}	1.05 ± 0.07^{aB}
	25	E+	14.85 ± 0.11^{aB}	1.76 ± 0.11^{aB}	3.90 ± 0.58^{bC}	1.04 ± 0.15^{aB}
		E−	14.63 ± 0.09^{aB}	1.68 ± 0.07^{aB}	5.34 ± 0.19^{aB}	1.00 ± 0.17^{aB}
	50	E+	14.66 ± 0.22^{aB}	1.72 ± 0.06^{aB}	4.34 ± 0.38^{abB}	1.04 ± 0.14^{aB}
		E−	15.20 ± 0.31^{aB}	1.72 ± 0.06^{aB}	4.57 ± 0.58^{abB}	1.05 ± 0.15^{aB}
28	0	E+	16.67 ± 0.42^{bcA}	3.58 ± 0.09^{aA}	5.40 ± 0.24^{abBC}	1.54 ± 0.23^{aA}
		E−	16.21 ± 0.27^{cA}	3.21 ± 0.05^{bcA}	7.01 ± 1.26^{aA}	1.52 ± 0.05^{aA}
	25	E+	16.75 ± 0.31^{bcA}	3.26 ± 0.07^{bA}	5.12 ± 0.19^{aB}	1.32 ± 0.07^{aA}
		E−	17.07 ± 0.59^{abA}	3.27 ± 0.16^{bA}	7.01 ± 1.26^{aA}	1.33 ± 0.14^{aA}
	50	E+	16.86 ± 0.50^{abcA}	3.05 ± 0.08^{cA}	5.07 ± 0.24^{aAB}	1.28 ± 0.10^{aA}
		E−	17.61 ± 0.30^{aA}	3.27 ± 0.06^{bA}	6.12 ± 0.84^{aA}	1.28 ± 0.22^{aA}

Notes.
E+ and E- represent endophyte-infected and non-infected plants, respectively. Data represent means $\pm$ standard error (SE). Means followed by different lowercase letters differ statistically among a given treatment time ($P < 0.05$). Means followed by different uppercase letters differ statistically under the same calcium nitrate treatment ($P < 0.05$).

Table 8 Spearman's rho correlation coefficients between indicators measured and temperature.

Parameter	Temperature
Root metabolic activity	0.734^{**}
Relative water content of leaf	0.761^{**}
Total chlorophyll	0.577^{*}
Chlorophyll a/ b ratio	0.492^{*}
Carotenoid	-0.203
Soluble sugar concentration of shoot	-0.564^{*}
Soluble sugar concentration of root	-0.944^{**}
Sucrose concentration of shoot	-0.682^{**}
Sucrose concentration of root	-0.564^{*}
Fructose concentration of shoot	-0.302
Fructose concentration of root	-0.013
Glucose concentration of Shoot	-0.394
Glucose concentration of root	-0.866^{**}

Notes.
*Correlation is significant at the 0.05 level.
**Correlation is significant at the 0.01 level.

Low temperature is known to reduce root vigor (*Sun et al., 2017*). Consistent with this, our results indicated that root metabolic activities of *F. sinensis* that were inhibited by low temperature decreased during prolonged cold stress (Fig. 2, Table 8). Endophyte infection was beneficial for root metabolic activity which was in agreement with results reported by *Liu et al. (2014)* who showed that cucumber inoculated with arbuscular mycorrhizal fungi increase root metabolic activity under low temperature. Further to this, the increase of root metabolic activity was very similar (on 0 d and 14 d) to several previous studies on cucumber and tomato treated with exogenous calcium (*Li, Gao & Liu, 2011*; *Yan et al., 2006*; *Liu et al., 2012*). Furthermore, endophytic fungi or exogenous calcium nitrate may be in effective in promoting root metabolic activity occurred early after cold treatment in field conditions (Fig. 2). In addition, our results showed that E- plants under $25Ca(NO_3)_2$ had higher root metabolic activity than E+25 on 28 d. It is thus likely that endophytes that inhabit aerial grass tissues of *F. sinensis* ineffectively enhanced the root metabolic activity under different $Ca(NO_3)_2$ treatments and low temperature. Ca concentration of roots in the present study need to be studied further.

Exogenous calcium supplied *in vitro* or arbuscular mycorrhiza or PRPP (*Pseudomonas* spp., *Burkholderia phytofirmans*) are capable of alleviating chlorophyll degradation in plants at low temperature (*Schaberg et al., 2011*; *Feng et al., 2010*; *Mishra et al., 2011*; *Zhou et al., 2012*; *Fernandez et al., 2012*; *Zhu, Song & Xu, 2009*). For example, arbuscular mycorrhiza enhanced pigment contents such as chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids in tomato plants subjected to normal or low temperature stress (*Latef & He, 2011*). However it has also been suggested that low carotenoid content was beneficial to pepper species exposed to low temperature and low irradiance (*Ou et al., 2015*). Similarly, *Song et al. (2015)* reported that waterlogging stress induced greater content of chlorophyll in E+ than in E- *Hordeum brevisubulatum*. *Zhou et al. (2012)* concluded that both AMF colonization and paclobutrazol were capable of alleviating the low-temperature damage by increasing chlorophyll synthesis. We found endophyte stimulated chlorophyll levels before treatment with calcium nitrate with inconsistent effects on the carotenoid levels through the time course (Table 3). This may be due to temperature variance affecting fluctuation of endophyte frequency in tall fescue tissue (*Ju et al., 2006*).

In the experiments described in this paper, RWC was significantly reduced with decreasing temperature (Table 8). However, endophyte infection increased the relative water content of leaves on the 14th day and 28th day (Fig. 3). It has been reported that RWC in mycorrhizal and non-mycorrhizal maize leaves are similar at all temperature treatments (*Zhu, Song & Xu, 2009*). One study has shown that inoculation with *Pseudomonas* strains significantly enhances root/shoot biomass, relative water content, chlorophyll, anthocyanin, proline, proteins, and amino acids, which are adaptations to cold stress in wheat (*Mishra et al., 2011*). These findings indicate that RWC may have been increased by microorganisms. Our results showed that the impact of 50 mM $Ca(NO_3)_2$ in reducing RWC was greater for E+ plant than for E- . *Epichloë* endophyte may be playing a role in nutrient uptake and osmotic adjustment.

Soluble carbohydrates appear essential in the response of plants to cold stress with elevated soluble sugar content (*Smith, 1975*; *Trom, Sheath & Bryant, 1989*; *Klotke et*

al., 2004; Stupnikova et al., 2002). We found significant correlation between soluble carbohydrates and temperature (Table 8). In species as diverse as perennial grass *Miscanthus*, a significant correlation between soluble sugars and minimum temperature has been reported (*Purdy et al., 2015*). The process of cold stress influences carbohydrate concentrations. *Saghfi & Eivazi (2014)* have found that glucose, rhamnose and mannose concentrations vary over time, with the maximum levels on the 6th day, and fructose contents continuously increase in susceptible chickpea whilst in the resistant cultivar glucose, rhamnose and mannose contents increased continuously and maximum levels of fructose were attained on the 4th day. Similarly, the dynamics of the changes in total soluble sugar, glucose, sucrose, sorbitol and fructose concentrations differ in two peach cultivars during the cold winter period (*Yooyongwech et al., 2009*). In the present study, soluble sugar and glucose concentrations of roots continued to increase as time passed, the maximum levels of fructose in shoots were achieved on the 14th day, but soluble sugars, sucrose, glucose concentrations of shoots and sucrose concentrations of roots were observed with maximum on the 28th day (Tables 6 and 7). The results suggest that cold affects the transformation of hexoses into sucrose and its subsequent splitting by invertase into hexoses again. In addition, many plant shoots accumulated more soluble sugars, fructose and glucose compared to roots, however on the 28th day sucrose concentrations in roots increased more than in shoots (Tables 6 and 7). Carbohydrate concentrations between oat and rye tissues have marked differences (*Livingston, Premakumar & Tallury, 2005*; *Livingston, Premakumar & Tallury, 2006*), which is consistent with our finding that soluble carbohydrate concentrations in shoots and roots varied. The concentration of 40 mg/g soluble sugar in *Miscanthus sacchariflorus* at Aberystwyth in November was larger than those observed only 9 mg/g at Harpenden (*Purdy et al., 2015*). These findings demonstrate that the soluble carbohydrate shifts are affected by many factors, such as sugar metabolism and transport, temperature, nutrient availability, stress time, plant tissue, site and species.

As previous works have noted, cold stress (4 °C) induces more carbohydrate accumulations such as starch, glucose, fructose, sucrose, mannose, raffinose and maltose, in grapevine inoculated with *Burkholderia phytofirmans* than in grapevine non-inoculated (*Fernandez et al., 2012*). Our data collected also suggested that *Epichloë* endophyte infection significantly increased soluble sugars of shoots with $25Ca(NO_3)_2$ and fructose of roots under the control conditions on the 14th day, and on the 28th day significantly increased soluble sugar of shoots with control and $25Ca(NO_3)_2$, glucose of shoots and sucrose of roots under the control conditions (Tables 6 and 7). Under low-temperature stress, *Zhu et al. (2015)* have found arbuscular mycorrhiza raise soluble sugars and reducing sugars of leaves, and sucrose and fructose concentrations of roots in maize, and a similar result is also reported by *Chen et al. (2014)*.

Although these studies have tested that microorganisms have an active role in the accumulation of sugars in the host under cold stress, they did not examine as $Ca(NO_3)_2$ application as in our study. Our results also indicated that calcium nitrate addition contributed to the accumulation of soluble sugars, sucrose, fructose or glucose in shoots and roots of both E+ and E- plants during long term cold stress (Tables 6 and 7). Calcium supplementation increases sugar concentrations such as fructose, sucrose, and glucose in

red spruce during both the fall and winter under field conditions (*Halman et al., 2008*; *Hawley et al., 2006*). It is thus likely that amylase activity has been induced by calcium (*Bush et al., 1989*; *Sadhukhan et al., 1990*; *Nielsen, Fuglsang & Westh, 2003*). As far as we know, this is the first study to report that calcium nitrate in combination with a fungal endophyte strongly increased soluble sugars in shoots (Tables 5 and 6).

In addition, *Soto-Barajas, et al. (2015)* suggested that *Epichloë* endophytes may affect nutrient absorption of *Lolium perenne* in field experiments and E+ had significantly lower Ca content in shoot at the flowering stage than E- plants. It thus is logical that supplementation with calcium would further improve plant performance. To the best of our knowledge, the results of the present study are the first evidence of a relationship between calcium nitrate and endophyte in *F. sinensis* exposed to cold field conditions.

CONCLUSIONS

In this work *F. sinensis* and associated *Epichloë* endophyte were used to study the effect of endophyte infection on physiological performance and soluble sugar concentrations under calcium nitrate and cold field conditions. *Epichloë* endophyte or $Ca(NO_3)_2$ significantly affected root metabolic activity, total chlorophyll, chlorophyll a/b ratio, and carotenoid. *Epichloë* endophyte and $Ca(NO_3)_2$ had no significant effect on chlorophyll a/b ratio. Some soluble sugar concentrations were affected by endophyte infection or interaction of the *Epichloë* endophyte and $Ca(NO_3)_2$ but not by $Ca(NO_3)_2$ alone. Further studies regarding soluble carbohydrate changes in addition to analysis of related enzyme activities, nutrient absorption and gene expression are necessary to elucidate the detailed mechanism of increased cold stress tolerance.

ACKNOWLEDGEMENTS

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

Experiment Station. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests

Richard D. Johnson is employed by AgResearch Limited of New Zealand. The authors declare there are no competing interests.

Author Contributions

- Lianyu Zhou conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Chunjie Li conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- James F. White and Richard D. Johnson analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

Raw data are available in the Supplementary Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.10568#supplemental-information.

REFERENCES

Bowler C, Fluhr R. 2000. The role of calcium and activated oxygens as signals for controlling cross-tolerance. *Trends in Plant Science* **5**:241–246 DOI 10.1016/s1360-1385(00)01628-9.

Burchett S, Niven S, Fuller MP. 2006. The effect of cold acclimation on the water relations and freezing tolerance of *Hordeum vulgare* L. *Cryo Letters* **27**:295–303.

Bush DS. 1995. Calcium regulation in plant cells and its role in signaling. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**:95–122 DOI 10.1146/annurev.pp.46.060195.000523.

Bush DS, Sticher L, Huystee Rvan, Wagner D, Jones RL. 1989. The calcium requirement for stability and enzymatic activity of two isoforms of barley aleurone α-amylase. *Journal of Biological Chemistry* **264**:19392–19398.

Chen N, He RL, Chai Q, Li CJ, Nan ZB. 2016. Transcriptomic analyses giving insights into molecular regulation mechanisms involved in cold tolerance by *Epichloë* endophyte in seed germination of *Achnatherum inebrians*. *Plant Growth Regulation* **80**:1–9 DOI 10.1007/s10725-016-0177-8.

Chen XY, Song FB, Liu FL, Tian CJ, Liu SQ, Xu HW, Zhu XC. 2014. Effect of different arbuscular mycorrhizal fungi on growth and physiology of maize at ambient and low temperature regimes. *The Scientific World Journal* 956141 DOI 10.1155/2014/956141.

Ding S, Huang CL, Sheng HM, Song CL, Li YB, An LZ. 2011. Effect of inoculation with the endophyte *Clavibacter* sp. strain Enf12 on chilling tolerance in *Chorispora bungeana*. *Physiologia Plantarum* **141**:141–151 DOI 10.1111/j.1399-3054.2010.01428.

Dong SF, Scagel CF, Cheng LL, Fuchigami LH, Rygiewicz PY. 2001. Soil temperature and plant growth stage influence nitrogen uptake and amino acid concentration of apple during early spring growth. *Tree Physiology* **21**:541–547 DOI 10.1093/treephys/21.8.541.

Feng Z, Liang F, Zheng CS, Shu HR, Sun XZ, Yoo YK. 2010. Effects of acetylsalicylic acid and calcium chloride on photosynthetic apparatus and reactive oxygen-scavenging enzymes in chrysanthemum under low temperature stress with low light. *Agricultural Sciences in China* **9**:1777–1786 DOI 10.1016/S1671-2927(09)60276-5.

Fernandez O, Theocharis A, Bordiec S, Feil R, Jacquens L, Clément C, Flontaine F, Barka EA. 2012. *Burkholderia phytofirmans* PsJN acclimates grapevine to cold by modulating carbohydrate metabolism. *Molecular Plant-Microbe Interactions* **25**:496–504 DOI 10.1094/MPMI-09-11-0245.

Halman J, Schaberg PG, Hawley GJ, Eagar C. 2008. Calcium addition at the Hubbard Brook Experimental Forest increases sugar storage, antioxidant activity and cold tolerance in native red spruce (*Picea rubens*). *Tree Physiology* **28**:855–862 DOI 10.1093/treephys/28.6.855.

Hansen J, Vogg G, Beck E. 1996. Assimilation, allocation and utilization of carbon by 3-year-old Scots pine (*Pinus sylvestris* L.) trees during winter and early spring. *Trees* **11**:83–90 DOI 10.1007/pl00009659855.

Hawley GJ, Schaberg PG, Eagar C, Borer C. 2006. Calcium addition at the Hubbard Brook Experimental Forest reduced winter injury to red spruce in a high-injury year. *Canadian Journal of Forest Research* **36**:2544–2549 DOI 10.1139/x06-221.

Hepler PK. 2005. Calcium: a central regulator of plant growth and development. *The Plant Cell* **17**:2142–2155 DOI 10.1105/tpc.105.032508.

Jeon J, Kim NY, Kim S, Kang NY, Novák O, Ku SJ, Cho C, Lee DJ, Lee EJ, Strnad M, Kim J. 2010. A subset of cytokinin two-component signaling system plays a role

in cold temperature stress response in *Arabidopsis. Journal Biological Chemistry* **285**:23371–23386 DOI 10.1074/jbc.M109.096644.

Johnson LJ, De Bonth ACM, Briggs LR, Caradus JR, Finch SC, Fleetwood DJ, Fletcher LR, Hume DE, Johnson RD, Popay AJ, Tapper BA, Simpson WR, Voisey CR, Card SD. 2013. The exploitation of *Epichloë* endophytes for agricultural benefit. *Fungal Diversity* **60**:171–188 DOI 10.1007/s13225-013-0239-4.

Ju HJ, Hill NS, Abbott T, Ingram KT. 2006. Temperature influences on endophyte growth in tall fescue. *Crop Science* **46**:404–412 DOI 10.2135/cropsci2005.0282.

Karpets YV, Kolupaev YE, Yastreb TO, Oboznyi AI. 2016. Induction of heat resistance in wheat seedlings by exogenous calcium, hydrogen peroxide, and nitric oxide donor: functional interaction of signal mediators. *Russian Journal of Plant Physiology* **63**:490–498 DOI 10.1134/S1021443716040075.

Klotke J, Kopka J, Gatzke N, Heyer AG. 2004. Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation evidence for a role of raffinose in cold acclimation. *Plant, Cell and Environment* **27**:1395–1404 DOI 10.1111/j.1365-3040.2004.01242.

Latch GCM, Hunt WF, Musgrave DR. 1985. Endophytic fungi affect growth of perennial ryegrass. *New Zealand Journal of Agricultural Research* **28**:165–168 DOI 10.1080/00288233.1985.10427011.

Latef AAHA, He CX. 2011. Arbuscular mycorrhizal influence on growth, photo-synthetic pigments, osmotic adjustment and oxidative stress in tomato plants subjected to low temperature stress. *Acta Physiologiae Plantarum* **33**:1217–1225 DOI 10.1007/s11738-010-0650-3.

Leuchtmann A, Bacon CW, Schardl CL, White JF, Tadych M. 2014. Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. *Mycologia* **106**:202–215 DOI 10.3852/13-251.

Li HS. 2000. *Principles and techniques of plant physiological biochemical experiment [M].* Beijing: Higher Education Press(in Chinese).

Li TL, Gao XQ, Liu YF. 2011. Effects of calcium on root activity and metabolism of reactive oxygen species of tomato seedlings under low night temperature stress. *Acta Agriculturae Boreali-occidentalis Sinica* **20**:127–132 (In Chinese, with English abstract).

Liu AR, Chen SC, Chang R, Liu DL, Chen HR. Ahammed GJ, Lin XM, He CX. 2014. Arbuscular mycorrhizae improve low temperature tolerance in cucumber via alterations in H_2O_2 accumulation and ATPase activity. *Journal of Plant Research* **127**:775–785 DOI 10.1007/s10265-014-0657-8.

Liu YF, Gao XQ, Qi MF, Li TL. 2012. Effect of calcium on production and accumulation of photosythate in tomato under low night temperature stress. *Jiangsu Journal of Agriculture Scinece* **28**:828–834 (In Chinese, with English abstract).

Livingston DP, Premakumar R, Tallury SP. 2005. Carbohydrate concentrations in crown fractions from winter oat during hardening at sub-zero temperatures. *Annals of Botany* **96**:331–335 DOI 10.1093/aob/mci167.

Livingston DP, Premakumar R, Tallury SP. 2006. Carbohydrate partitioning between upper and lower regions of the crown in oat and rye during cold acclimation and freezing. *Cryobiology* **52**:200–208 DOI 10.1016/j.cryobiol.2005.11.001.

Lootens P, Van Waes J, Carlier L. 2004. Effect of a short photoinhibition stress on photosynthesis, chlorophyll a fluorescence, and pigment contents of different maize cultivars. Can a rapid and objective stress indicator be found? *Photosynthetica* **42**:187–192 DOI 10.1023/B:PHOT.0000040589.09614.a0001.

Ma MZ, Christensen MJ, Nan ZB. 2015. Effects of the endophyte *Epichloë festucae* var. *lolii* of perennial ryegrass (*Lolium perenne*) on indicators of oxidative stress from pathogenic fungi during seed germination and seedling growth. *European Journal of Plant Pathology* **141**:571–583 DOI 10.1007/s10658-014-0563-xa0001.

Min HL, Cai SJ, Rui Z, Sha S, Xie KB, Xu QS. 2012. Calcium-mediated enhancement of copper tolerance in *Elodea canadensis*. *Biologia Plantarum* **57**:365–369 DOI 10.1007/s10535-012-0275-zxa0001.

Mishra PK, Bisht SC, Ruwari P, Selvakumar G, Joshi GK, Bisht JK, Bhatt J, Gupta HS. 2011. Alleviation of cold stress in inoculated wheat (*Triticum aestivum* L.) seedlings with psychrotolerant *Pseudomonads* from NW Himalayas. *Archives of Microbiology* **193**:497–513 DOI 10.1007/s00203-011-0693.

Monroy AF, Sarhan F, Dhindsa R. 1993. Cold-induced changes in freezing tolerance, protein phosphorylation, and gene expression (evidence for a role of calcium). *Plant Physiology* **102**:1227–1235 DOI 10.1104/pp.102.4.1227.

Nan ZB, Li CJ. 2000. Neotyphodium in native grasses in China and observations on endophyte/host interactions. In: *Proceedings of the 4th international Neotyphodium/-grass interactions symposium*. Soest, Germany.

Navarro JM, Martínez V, Carvajal M. 2000. Ammonium, bicarbonate and calcium effects on tomato plants grown under saline conditions. *Plant Science* **157**:89–96 DOI 10.1016/S0168-9452(00)00272-7.

Nayyar H, Bains T, Kumar S. 2005. Low temperature induced floral abortion in chickpea: relationship to abscisic acid and cryoprotectants in reproductive organs. *Environmental and Experimental Botany* **53**:39–47 DOI 10.1016/j.envexpbot.2004.02.011bj20030220.

Nielsen AD, Fuglsang CC, Westh P. 2003. Effect of calcium ions on the irreversible denaturation of a recombinant *Bacillus halmapalus* alpha-amylase: a calorimetric investigation. *Biochemical Journal* **373**:337–343 DOI 10.1042/bj20030220.

Ou LJ, Wei G, Zhang ZQ, Dai XZ, Zou XX. 2015. Effects of low temperature and low irradiance on the physiological characteristics and related gene expression of different pepper species. *Photosynthetica* **53**:85–94 DOI 10.1007/s11099-015-0084-7.

Peng QQ, Li CJ, Song ML, Nan ZB. 2013. Effects of seed hydropriming on growth of *Festuca sinensis* infected with *Neotyphodium* endophyte. *Fungal Ecology* **6**:83–91 DOI 10.1016/j.funeco.2012.08.001.

Purdy SJ, Cunniff J, Maddison AL, Jones LE, Barraclough T, Castle M, Davey CL, Jones CM, Shield I, Gallagher J, Donnison I, Clifton-Brown J. 2015. Seasonal

carbohydrate dynamics and climatic regulation of senescence in the perennial grass, *Miscanthus*. *Bioenergy Research* **8**:28–41 DOI 10.1007/s12155-014-9500-2.

Rahman A, Mostofa MG, Nahar K, Hasanuzzaman M, Fujita M. 2016. Exogenous calcium alleviates cadmium-induced oxidative stress in rice (*Oryza sativa* L.) seedlings by regulating the antioxidant defense and glyoxalase systems. *Brazilian Journal of Botany* **39**:393–407 DOI 10.1007/s40415-015-0240-0.

Redman RS, Kim YO, Woodward CJDA, Greer CA, Espino L, Doty SL, Rodriguez RJ. 2011. Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. *PLOS ONE* **6**:e14823 DOI 10.1371/journal.pone.0014823.

Rodriguez R, Redman R. 2008. More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *Journal of Experimental Botany* **59**:1109–1114 DOI 10.1093/jxb/erm342.

Rozpadek P, Wezowicz K, Nosek M, Wazny R, Tokarz K, Lembicz M, Miszalski Z, Turnau K. 2015. The fungal endophyte *Epichloë typhina* improves photosynthesis efficiency of its host orchard grass (*Dactylis glomerata*). *Planta* **242**:1025–1035 DOI 10.1007/s00425-015-2337-x.

Sadhukhan R, Manna S, Roy SK, Chakrabarty SL. 1990. Thermostable amylolytic enzymes from a cellulolytic fungus *Myceliophthora thermophila* D14 (ATCC48104). *Applied Microbiology and Biotechnology* **33**:692–696 DOI 10.1007/BF00604940.

Saghfi S, Eivazi AR. 2014. Effects of cold stress on proline and soluble carbohydrates in two chickpea cultivars. *International Journal of Current Microbiology and Applied Sciences* **3**:591–595.

Shanghai Institute of Plant Physiology Chinese Academy of Sciences. 1999. *Guide of modern plant physiological experiment [M]*. Beijing: Science Press(in Chinese).

Schaberg PG, Minocha R, Long S, Halman JM, Hawley GJ, Eagar C. 2011. Calcium addition at the Hubbard Brook Experimental Forest increases the capacity for stress tolerance and carbon capture in red spruce (*Picea rubens*) trees during the cold season. *Trees* **25**:1053–1061 DOI 10.1007/s00468-011-0580-8.

Schmitz-Eiberger M, Haefs R, Noga G. 2002. Calcium deficiency-influence on the antioxidative defense system in tomato plants. *Journal of Plant Physiology* **159**:733–742 DOI 10.1078/0176-1617-0621.

Shi HT, Ye TT, Zhong B, Liu X, Chan ZL. 2014. Comparative proteomic and metabolomic analyses reveal mechanisms of improved cold stress tolerance in bermudagrass (*Cynodon dactylon* (L.) Pers.) by exogenous calcium. *Journal of Integrative Plant Biology* **56**:1064–1079 DOI 10.1111/jipb.12167.

Smith D. 1975. Influence of temperature on growth of Froker oats for forage. II. Concentrations and yields of chemical constituents. *Canada Journal of Plant Science* **55**:897–901 DOI 10.4141/cjps75-143.

Song ML, Li XZ, Saikkonen K, Li CJ, Nan ZB. 2015. An asexual *Epichloë* endophyte enhances waterlogging tolerance of *Hordeum brevisubulatum*. *Fungal Ecology* **13**:44–52 DOI 10.1016/j.funeco.2014.07.004.

Soto-Barajas MC, Zabalgogeazcoa I, Gómez-Fuertes J, González-Blanco V, Vázquez-de-Aldana BR. 2015. *Epichloë* endophytes affect the nutrient and fiber content of *Lolium perenne* regardless of plant genotype. *Plant Soil* **405**:265–277 DOI 10.1007/s11104-015-2617.

Stupnikova IV, Borovskii GB, Dorofeev NV, Peshkova AA, Voinikov VK. 2002. Accumulation and disappearance of dehydrins and sugars depending on freezing tolerance of winter wheat plants at different developmental phases. *Journal of Thermal Biology* **27**:55–60 DOI 10.1016/S0306-4565(01)00015-8.

Sun B, Liu GL, Phan TT, Yang LT, Li YR, Xing YX. 2017. Effects of cold stress on root growth and physiological metabolisms in seedlings of different sugarcane varieties. *Sugar Tech* **19**:165–175 DOI 10.1007/s12355-016-0452-z.

Tan W, Meng QW, Brestic M, Olsovska K, Yang XH. 2011. Photosynthesis is improved by exogenous calcium in heat-stressed tobacco plants. *Journal of Plant Physiology* **168**:2063–2071 DOI 10.1016/j.jplph.2011.06.009.

Trom ER, Sheath GW, Bryant AM. 1989. Seasonal variations in total nonstructural carbohydrate and major element levels in perennial ryegrass and paspalum in a mixed pasture. *New Zealand Journal of Agricultural Research* **32**:157–165 DOI 10.1080/00288233.1989.10423450.

Wang C, Wang C, Gao YL, Wang YP, Guo JH. 2016. A consortium of three plant growth-promoting rhizobacterium strains acclimates *Lycopersicon esculentum* and confers a better tolerance to chilling stress. *Journal of Plant Growth Regulation* **35**:54–64 DOI 10.1007/s00344-015-9506-9.

Xia C, Li NN, Zhang XX, Feng Y, Christensen MJ, Nan ZB. 2016. An *Epichloë* endophyte improves photosynthetic ability and dry matter production of its host *Achnatherum inebrians* infected by *Blumeria graminis* under various soil water conditions. *Fungal Ecology* **22**:26–34 DOI 10.1016/j.funeco.2016.04.002.

Xu C, Li X, Zhang L. 2013. The effect of calcium chloride on growth, photosynthesis, and antioxidant responses of *Zoysia japonica* under drought conditions. *PLOS ONE* **8**:e68214 DOI 10.1371/journal.pone.0068214.

Yan T, Wang XF, Yang FJ, Wei M. 2006. Effect of calcium on chilling resistance of cucumber seedlings under root zone low temperature stress. *Acta Agriculturae Boreali-occidentalis Sinica* **15**:172–176 , 181 (In Chinese, with English abstract).

Yang SL, Lan SS, Deng FF, Gong M. 2016. Effects of calcium and calmodulin antagonists on chilling stress-induced proline accumulation in *Jatropha curcas* L. *Journal of Plant Growth Regulation* **35**:815–826 DOI 10.1007/s00344-016-9584-3.

Yang S, Wang F, Guo F, Meng JJ, Li XG, Dong ST, Wan SB. 2013. Exogenous calcium alleviates photoinhibition of PSII by improving the xanthophyll cycle in peanut (*Arachis hypogaea*) leaves during heat stress under high irradiance. *PLOS ONE* **8**:e71214 DOI 10.1371/journal.pone.0071214.

Yao X, Li XZ, Zhu XX, Li CJ. 2013. Effects of two fungicides on *Neotyphodium* seed-borne fungal endophyte of *Festuca sinensis*. *Pratacultural Science* **10**:1517–1522 (in Chinese, with English abstract).

Yin L, Ren A, Wei M, Wu L, Zhou Y, Li X, Gao Y. 2014. *Neotyphodium coenophialum-* infected tall fescue and its potential application in the phytoremediation of saline soils. *International Journal of Phytoremediation* **16**:235–246 DOI 10.1080/15226514.2013.773275.

Yooyongwech S, Sugaya S, Sekozawa Y, Gemma H. 2009. Differential adaptation of high- and low-chill dormant peaches in winter through aquaporin gene expression and soluble sugar content. *Plant Cell Reports* **28**:1709–1715 DOI 10.1007/s00299-009-0770-7.

Zhou B, Guo Z. 2009. Calcium is involved in the abscisic acid-induced ascorbate peroxidase, superoxide dismutase and chilling resistance in *Stylosanthes guianensis*. *Biologia Plantarum* **53**:63–68 DOI 10.1007/s10535-009-0009-z.

Zhou LY, Li CJ, Zhang XX, Johnson R, Bao GS, Yao X, Chai Q. 2015a. Effects of cold shocked *Epichloë* infected *Festuca sinensis* on ergot alkaloid accumulation. *Fungal Ecology* **14**:99–104 DOI 10.1016/j.funeco.2014.12.006.

Zhou LY, Zhang XX, Li CJ, Christensen MJ, Nan ZB. 2015b. Antifungal activity and phytochemical investigation of the asexual endophyte of *Epichloë* sp. from *Festuca sinensis*. *Science China Life Sciences* **8**:821–826 DOI 10.1007/s11427-015-4845-0.

Zhou ZZ, Ma HM, Liang KN, Huang GH, Pinyopusarerk K. 2012. Improved tolerance of teak (*Tectona grandis* L.f.) seedlings to low-temperature stress by the combined effect of arbuscular mycorrhiza and paclobutrazol. *Journal of Plant Growth Regulation* **31**:427–435 DOI 10.1007/s00344-011-9252-6.

Zhu XC, Song FB, Liu FL, Liu SQ, Tian CJ. 2015. Carbon and nitrogen metabolism in arbuscular mycorrhizal maize plants under low-temperature stress. *Crop and Pasture Science* **66**:62–70 DOI 10.1071/cp14159.

Zhu XC, Song FB, Xu HW. 2009. Arbuscular mycorrhizae improves low temperature stress in maize via alterations in host water status and photosynthesis. *Plant Soil* **331**:129–137 DOI 10.1007/s11104-009-0239.