Effects of interaction of Epichloë gansuensis and Bacillus strains on the seed germination and seedling growth in Achnatherum inebrians plants

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Abstract

Previous studies have confirmed that *Bacillus* and also *Epichloë* fungal endophytes can promote seed germination of grasses and induce stress resistance. However, the effects of interactions between *Bacillus*and endophyte on seed germination and seedling growth of grasses have not been well studied. In this study, *Epichloë gansuensis* endophyte-infected and endophyte-free *Achnatherum inebrians* seeds, and two month old seedlings, were inoculated with 12 *Bacillus* strains, and then effects of the treatments on four germination indicators and eight growth parameters were determined. The results showed that the interaction between *E. gansuensis* and *Bacillus* strains significantly (P<0.05) increased seed germination and the growth of *A. inebrians.* Interestingly, *Bacillus* strain inoculation, except for strain B96, provided a greater increase in growth on the 2-3 month old seedlings than the increase of growth with young seedlings. The germination rate of *A. inebrians*inoculated with the 12 strains ranged from 93.7% to 99%. Compared with other strains, B18 and B419 showed greater increases in growth of 2-3 months old plants. Moreover, the study employed a membership function value to assess the growth-promoting ability of the 12 *Bacillus*strains on *A. inebrians* as an integrative tool for screening strains. Among the 12 strains, the B419 strain showed the best growth-promoting ability, while B106 was the worst strain. These results indicate that the B419 strain has potential value in agricultural production, and mechanisms of plant growth promotion will be further studied.

1 Introduction

Epichloë endophytes that are symbiotic with many cool season grasses spend all or part of their life cycle in the host grass without external symptoms (Schardl et al. 2004). At present, 49 species of *Epichloë* endophytes have been isolated and identified (Leuchtmann et al. 2014; Chen et al. 2015; Shymanovich et al. 2017; Leuchtmann et al. 2019; Tian et al. 2020; Thuenen et al. 2022). There are 10 species that were found by Chinese scholars (Song et al. 2015; Tian et al. 2020). Studies on grass - *Epichloë* endophyte associations are mainly focused on *Lolium* and *Festuca* plants (Johnson et al. 2013; Leuchtmann et al. 2014). These mutualistic fungal endophytes not only enhance host plant resistance to biotic and abiotic stresses by promoting plant growth, as well as nutrient use efficiency and photosynthesis (Song et al. 2015; Xia et al. 2018b; Wang et al. 2019), but also produce alkaloids that are toxic to livestock, thereby deterring grazing, and alkaloids that are toxic or are feeding deterrents of insects, and enhancing persistence and productivity of host plants (Johnson et al. 2013, Liang et al. 2017).

Achnatherum inebrians is a perennial bunchgrass, which mainly grows on the arid and semi-arid grasslands in the north and northwest of China (Li et al. 2004; Xia et al. 2018a). A previous study found that the frequency of endophyte infection of *A. inebrians* was nearly 100% in the native grassland (Nan and Li 2000). The endophytes isolated from *A. inebrians* are either *Epichloë gansuensis* (Li et al. 2004; Leuchtmann et al. 2014) or *E. inebrians* (Chen et al. 2015). Liang et al. (2017) reported the symbiosis of *A. inebrians* and endophyte could produce alkaloids (ergonovine and ergine), which leads to livestock poisoning. Some 30 years of research on the symbiosis of *A. inebrians* and *Epichloë* endophytes have found that their presence could enhance host grasses resistance to heavy metals (Zhang et al. 2010a, b), low temperatures (Chen et al. 2016), drought (Xia et al. 2018a; Zhong et al. 2021; Zhao et al. 2021, 2022), salinity (Wang et al. 2019; Ju et al. 2021), pathogens (Xia et al. 2015, 2016; Kou et al. 2021) and insects (Zhang et al. 2012). In addition, with the increase of soil moisture, the fungal diversity and bacterial richness in the rhizosphere soil increased, while the root fungal diversity decreased (Zhong et al. 2018; Ju et al. 2020). Subsequently, Liu et al. (2022) found that the presence of an *Epichloë* endophyte increased the diversity of the phyllosphere bacterial and fungal communities in *A. inebrians*, and the diversity and the richness of the endophytic bacterial and fungal communities were different from those of the epiphytic microbes community.

Bacillus is a gram-positive, rod-shaped, aerobic or facultative anaerobic bacterial genus, which can produce stress-resistant endospores (Hong et al. 2005). This genus comprises about 200 species of bacteria, which are almost ubiquitous in nature, such as in plants, soil, air and water environments (Soltani et al. 2019; Gopta et al. 2020). Up to now, *B. subtilis* (Xu et al. 2022), B. *amyloliquefaciens* (Jiao et al. 2020), B. *megaterium* (Antil et al. 2022), B. *cereus* (Antil et al. 2022), B. *altitudinis* (Zhang et al. 2021), B. *thuringiensis* (Liang et al. 2022)d *licheniformis* (Muthuraja and Muthukumar 2022) have been utilized in agricultural production. *Bacillus* spp. can promote plant growth through a variety of mechanisms, including increased nutrient uptake by the plant mediated solubilization of insoluble forms (Wang et al. 2022), fixation of nitrogen (Franco-Sierra et al. 2020), production of plant hormones to regulate plant growth (Akhtar et al. 2021), or production of volatile organic compounds (VOCs) (Tahir et al. 2017). In addition, strains of this genus limit the infection of plants by pathogens by secreting bacteriostatic substances or inducing systemic plant resistance (Poveda et al. 2020; Poveda and Gonzalez-Andres 2021).

Seed is the basis of reproduction and dispersion of spermatophytes (Ding et al. 2022). Seed germination is an indispensable stage for initiating seedling establishment and subsequent plant growth (Yu et al. 2021), and plays a critical role in the proliferation of plants, efficient crop production, and a successful crop improvement research program (Maeda et al. 2021). Germination is affected by abiotic factors such as light, temperature, planting depth, drought stress, salt stress and seed secretion, as well as biological factors such as plant growth-promoting or biocontrol bacteria, plant pathogens and commensal microbes (Barret et al. 2016; Lamichhane et al. 2018; Yue et al. 2021). A large number of studies have proved that *Epichloë* endophytes improve the ability of host plants to resist heavy metal (Zhang et al. 2010b), low temperature (Chen et al. 2016), drought (Bu et al. 2018), salt (Wang et al. 2019), and pathogen (Ma et al. 2015; Li et al. 2017) stresses at the germination stage. Interestingly, Bao et al. (2019) found that compared with the *A. inebrians* seeds collected from desert and arid grassland origins, the germination rate of the seeds collected from alpine regions was higher at low temperatures. Chen et al. (2021) showed that the interaction between *Epichloë* endophyte and warm water soaking treatment increased the germination of *A. inebrians* seeds from the Xiahe site, with seedlings having increased content of soluble sugar, IAA, and GA.

In a previous study, our research team isolated *Bacillus* from rhizosphere soil and roots of *A. inebrians*, and found that endophyte infection affected the diversity of *Bacillus* in rhizosphere soil and roots of *A.*

inebrians plants growing in field plots (Ju et al. 2021). The effect of *Bacillus* on plant growth and stress tolerance has been well documented (Rizvi et al. 2019; Zubair et al. 2019; Mendoza-Labrador et al. 2021; Ju et al. 2021; Ayaz et al. 2022; Xie et al. 2022), but the effects of the genus on seed germination and seedling growth of *A. inebrians* have not been reported. In the present study, through the seed germination experiment and greenhouse experiment, 12 strains of *Bacillus* were inoculated onto the seeds and seedlings of EI and EF *A. inebrians* to examine the following questions: (i) Do endophyte, *Bacillus* and their interactions affect seed germination of *A. inebrians*? (ii) What is the influence of endophyte, *Bacillus* and their interactions on the growth of *A. inebrians* at seedling and mature stages?

2 Materials And Methods

2.1 Materials

2.1.1 Seeds origin

The seeds used in the present experiments were collected from fields of endophyte-infected (EI) and endophyte-free (EF) *A. inebrians* plants in Yuzhong campus of Lanzhou University (104°390'E, 35°890'N, Altitude 1653 m), which were established in 2012 (Kou et al. 2021). In order to determine the fungal endophyte infection frequency of the seeds, the stems and seeds of *A. inebrians* collected from the above experimental fields were stained with aniline blue and then examined by microscopy (Zhong et al. 2022). The frequency of fungal endophyte of EI and EF seeds were 100% and 0%.

2.1.2 *Bacillus* strains origin

The *Bacillus* strains for the experiment were isolated from the roots, rhizosphere soil, and bulk soil from the *A. inebrians* experimental field. There were 12 strains used in this study (Table 1).

Table 1
Bacillus strains strains

Serial number	Strain number	Strain origin
1	B4	Rhizosphere soil
2	B9	Root
3	B10	Root
4	B15	Root
5	B18	Root
6	B96	Root
7	B106	Root
8	B154	Root
9	B181	Bulk soil
10	B382	Bulk soil
11	B419	Rhizosphere soil
12	B453	Bulk soil

2.2 Method

2.2.1 Preparation of bacterial solutions

The *Bacillus* strains were recovered from samples stored in the microbiological incubator and subsamples were inoculated into Luria broth (LB) medium sterilized at 121°C for 30 min, and incubated at 28°C on a constant temperature shaker with 180 rpm shaking, up to the logarithmic growth period. Then the cells were collected by 2 min centrifugation at 12000 rpm, and the supernatant was discarded. The cells were re-suspended in sterile LB medium to the density of OD_{600} = 0.9 and were used for subsequent inoculation experiments (Ju et al. 2021).

2.2.2 Germination experiment

Referring to the method of Ju et al. (2021), EI and EF *A. inebrians* seeds were first surface-sterilized with 75% alcohol for 1 min, then immersed in 2% NaClO for 10 min, followed by six rinses with sterile water and then dried on sterilized filter paper. The sterilized EI and EF seeds were immersed for five minutes in solutions of each of 12 *Bacillus* strains with an OD_{600} value of 0.8, respectively, and the control (CK) seeds were placed in sterilized LB medium. After soaking, the seeds were placed in Petri plates containing two layers of filter paper moistened with sterile water, 50 seeds per plate, 4 replicates for each treatment, and incubated in a constant temperature incubator (DHP9082A, Shanghai) at 25 ± 2°C, with 16 h of illumination and 8 h of darkness; the relative humidity was about 70 ± 10%. Sterile water was replenished every 24 h during the experiment.

Germination energy and germination rate, seedling length and radicle length were recorded at the end of the 21-day experiment.

Germination rate (GR) = (Gt/T)*100%, where T is the total number of seeds, and Gt is the number of germinated seeds on the 14th day of germination. Germination energy (GE), which reflects the speed and consistency of seed germination (Domin et al. 2020), is the germination rate on the 4th day of germination according to the ISTA (2006) standard. At the end of seed germination experiment, the seedlings were carefully taken out from the Petri dish and the shoot and radicle lengths were measured. All seedlings in each Petri dish were measured, and then the mean was calculated.

2.2.3 Greenhouse experiment

The plants used in the greenhouse pot experiment were cultivated in a controlled environment greenhouse (temperature 26 ± 2°C, the relative humidity 42 ± 2%) at the Chengguan Campus of Lanzhou University. Into each of 120 pots (15 cm in diameter and 16 cm in height), in which two layers of filter paper had been placed, 600g of a 2:1 vermiculite and black soil mixture that had been autoclaved at 121°C for 5 h, was added. The EI and EF seeds (same as used for the germination study) were sown in 60 pots respectively, with 3 seeds in each pot. After one month of growth, the pots were thinned to a single seedling. After the second fully unfolded leaf appeared, the plants were regularly watered with Hoaglands nutrient solution. In order to observe the effect of *Bacillus* on the growth of *A. inebrians* at the mature stage, 52 pots EI and 52 pots EF plants grown in pots for two months were treated with bacterial solution, 4 replicates for each of the 12 *Bacillus* strains. After one month the root length, plant height, aboveground dry/fresh weight, belowground dry/fresh weight, and chlorophyll content were measured.

Chlorophyll content was determined by the method of Xia et al. (2018), and was measured using a handheld chlorophyll meter (SPAD-502Plus, Konica Minolta Sensing, Inc., Japan). The tallest leaf of each plant was selected and the average of the four measurements was used as the relative value of chlorophyll content (SPAD value) for that plant. The SPAD values were converted to plant-specific chlorophyll content by referring to the method of Ye et al. (2017). $Y(mg/dm2) = 0.0996X - 0.15$ (X is the plant SPAD value and Y is the plant specific chlorophyll content).

2.3 Data analysis

Before the analysis, the germination data was tested in an arcsine transformation. The significance of the experimental data was determined using SPSS18.0 statistical analysis software (SPSS, Chicago, IL, USA). According to the fuzzy affiliation function method (Wu et al. 2019), different strains were comprehensively evaluated for their ability to promote the growth of *A. inebrians* plants, so as to screen out the superior strains. Firstly, the membership function value (MFV) of corresponding measured parameters of each strain was calculated, and then the average affiliation function value of each strain was calculated, and the larger the value, the stronger the growth-promoting ability of the strain. Significance is defined at the 95% confidence level.

$MFV = (X - Xmin)/(Xmax - Xmin)$. Where X is the measured value of corresponding parameter for each strain treatment, and Xmax and Xmin are the minima and maxima of corresponding parameters of 12 strain treatments.

3 Results

3.1 Germination energy and rate

The germination energy of *A. inebrians* seeds was significantly (P < 0.05) affected by *Bacillus* strains (F = 6.268, P < 0.000), *Epichloë* endophyte (F = 6.416, P = 0.014) and their interaction (F = 2.372, P = 0.016) (Table 2). The germination energy of EI *A. inebrians* was significantly (P < 0.05) higher than that of EF plants after inoculation with B154 strain (Fig. 1a). The germination energy of B154(EI), B181(EI), B382(EI), B419(EF), and B453(EF) *A. inebrians* seeds was significantly (P < 0.05) higher than that of seeds of CK plants (Fig. 1a).

Table 2 Two-way analysis of variance (ANOVA) for the effects of presence or absence of the *Epichloë* endophyte (E) and *Bacillus* (B) treatments on seeds germination of *A. inebrians*

Treatment	df	Germination energy (%)		Germination rate (%)		Radical Length (cm)		Seedling Length (cm)	
		F	P	F	P	F	P	F	P
E	1	6.416	**0.014**	1.833	0.182	15.495	**0.000**	29.631	**0.000**
B	12	6.268	**0.000**	3.429	**0.001**	7.674	**0.000**	4.326	**0.000**
E×B	12	2.372	**0.016**	0.652	0.788	2.140	**0.030**	3.681	**0.000**

Inoculation with *Bacillus* strains (F = 3.429, P < 0.010) had significant (P < 0.05) effects on germination rate of *A. inebrians* seeds, but *Epichloë* endophyte infection (F = 1.833, P = 0.812) and the interaction between *Bacillus* strain and *Epichloë* endophyte (F = 0.652, P = 0.0788) had no significant difference (Table 2). Compared with the CK, inoculation of B419 significantly (P < 0.05) increased the germination rate of *A. inebrians* seeds, up to 99% (Fig. 1b).

3.2 Length of seedling and radicle

The seedling length was significantly (P < 0.05) affected by *Bacillus* strains inoculation (F = 4.326, P < 0.000), *Epichloë* endophyte infection (F = 29.631, P < 0.000), and their interaction (F = 3.681, P < 0.000) (Table 2). The seedling length of EI *A. inebrians* was significantly (P < 0.05) greater than that of EF plants after inoculation with B10, B15, and B419 (Fig. 2a). The seedling length of B4(EI), B9(EI), B10(EI), B15(EI), B181(EF), and B419(EI) *A. inebrians* was significantly (P < 0.05) greater than that of CK plants (Fig. 2a).

There were significant (P < 0.05) differences on the radicle length with *Bacillus* strains inoculation (F = 7.674, P < 0.000), *Epichloë* endophyte infection (F = 15.495, P < 0.000) and their interaction (F = 2.372, P = 0.030) (Table 2). The radicle length of EI *A. inebrians* was significantly (P < 0.05) greater than that of EF seedlings after inoculation with B10 and B106 (Fig. 2b). The radicle length of B10(EI), B18(EI), B18(EF), and B419(EI) *A. inebrians* was significantly ($P < 0.05$) greater than that of CK seedlings (Fig. 2b).

3.3 Plant height, root length, tiller number and chlorophyll content

Inoculation of two-month-old plants with *Bacillus* strains ($F = 16.228$, $P < 0.000$) and the interaction between *Bacillus* strains and *Epichloë* endophyte ($F = 6.629$, $P < 0.000$) had significant ($P < 0.05$) effects on plant height (Table 3). Compared with the CK, inoculated with B4, B9, B18, B154, B181 and B419 all significantly ($P < 0.05$) increased the plant height, while the treatments of B10, B15, and B106 strains showed significant ($P < 0.05$) inhibitory effects (Fig. 3a).

Table 3 Two-way analysis of variance (ANOVA) for the effects of presence or absence of the *Epichloë* endophyte (E) and *Bacillus* (B) treatments on plant growth of *A. inebrians*

Response variable	Treatments	Df	F-value	P-value
Plant height(cm)	E	1	1.356	0.250
	B	12	16.228	**0.000**
	E xB	12	6.629	**0.000**
Root length(cm)	E	1	9.754	**0.003**
	B	12	12.900	**0.000**
	E xB	12	6.503	**0.000**
Tiller number(*plant-1)	E	1	0.381	0.540
	B	12	16.067	**0.005**
	E xB	12	2.806	**0.000**
Shoot fresh weight(g)	E	1	8.829	**0.004**
	B	12	26.885	**0.000**
	E xB	12	2.139	**0.030**
Root fresh weight(g)	E	1	23.814	**0.000**
	B	12	15.221	**0.000**
	E xB	12	4.201	**0.000**
Shoot dry weight(g)	E	1	0.002	0.969
	B	12	54.498	**0.000**
	E xB	12	6.419	**0.000**
Root dry weight(g)	E	1	6.419	**0.0 00**
	B	12	14.812	**0.000**
	E xB	12	3.245	**0.002**
Chlorphyll content	E	1	18.909	**0.000**
	B	12	65.686	**0.000**
	E xB	12	0.455	0.931

Inoculation with *Bacillus* strains ($F = 12.900$, $P < 0.000$), *Epichloë* endophyte infection ($F = 9.754$, $P = 0.003$), and their interactions ($F = 6.503$, $P < 0.000$), all had significant ($P < 0.05$) effects on root length (Table 3). The root length of EI *A. inebrians* was significantly ($P < 0.05$) greater than that of EF plants under inoculation with B4, B96, B181, B382, B419 and B453 (Fig. 3b). Except for B4(EF), B96(EF), B106(EI), B106(EF), B181(EF), and B453(EF), the other treatments all significantly ($P < 0.05$) increased the root length of *A. inebrians* (Fig. 3b).

Inoculation with *Bacillus* strains (F = 16.067, P = 0.005) and the interaction between *Bacillus* strains and *Epichloë* endophyte (F = 2.806, P < 0.000) had significant (P < 0.05) effects on tiller numbers (Table 3). Compared with the CK, except for the B10, B106, and B453, other strains all significantly (P < 0.05) promoted the tiller numbers of *A. inebrians* (Fig. 3c). The tiller numbers of *A. inebrians* plants inoculated with strain B419 were significantly (P < 0.05) higher than that of other treatments (Fig. 3c).

Epichloë endophyte infection (F = 18.909, P < 0.000) and inoculation with *Bacillus* strains (F = 65.686, P < 0.000) significantly (P < 0.05) affected chlorophyll content (Table 3). Compared with the CK, the *Bacillus* strains, except for strain B96, significantly (P < 0.05) increased chlorophyll content of *A. inebrians* (Fig. 3d). After inoculation with B18 and B106, the chlorophyll content of EI *A. inebrians* was significant (P < 0.05) higher than of EF plants (Fig. 3d). The chlorophyll content of *A. inebrians* inoculated with B18 strain was significantly (P < 0.05) higher than that of other treatments (Fig. 3d).

3.4 Fresh and dry weight

The shoot fresh weight of *A. inebrians* was significantly (P < 0.05) affected by the treatment of *Bacillus* strains (F = 26.885, P = 0.000), *Epichloë* endophyte infection (F = 8.829, P = 0.004) and their interaction (F = 2.139, P = 0.030) (Table 3). After inoculation with strains B18 and B419, the shoot fresh weight of EI *A. inebrians* was significantly (P < 0.05) higher than of EF plants (Fig. 4a). Except for B4(EF), B9(EF), B10(EF), B106(EI), B106(EF), B453(EI), and B453(EF), the other treatments all significantly (P < 0.05) increased the shoot fresh weight of *A. inebrians* (Fig. 4a).

Bacillus strains inoculation (F = 54.498, P < 0.000) and the interaction between *Bacillus* strains and *Epichloë* endophyte (F = 6.419, P < 0.000) had significant (P < 0.05) effects on shoot dry weight (Table 3). Compared with the CK, except for the strain B106, other strains all significantly (P < 0.05) enhanced the shoot dry weight of EI and EF *A. inebrians* (Fig. 4b). The shoot dry weight of EI *A. inebrians* was significantly (P < 0.05) higher than of EF plants under strain B419 treatment, while the shoot dry weight of EI *A. inebrians* was significantly (P < 0.05) lower than of EF plants under B106 B181, and B453 treatments (Fig. 4b).

There were significant (P < 0.05) differences on root fresh weight with *Bacillus* inoculation (F = 15.221, P < 0.000), *Epichloë* endophyte infection (F = 23.814, P < 0.000) and their interaction (F = 4.202, P < 0.000) (Table 3). The root fresh weight of EI *A. inebrians* was significantly (P < 0.05) higher than of EF plants under B15, B18, B96, and B419 treatments (Fig. 4c). The root fresh weight of B15(EI), B18(EI), B18(EF), B96(EI), B154(EI), B419(EI), and B419(EF) was significantly (P < 0.05) higher than of CK plants, and the root fresh weight of B18(EI) was significantly (P < 0.05) higher than B18(EF), B154(EI), and B419(EF) plants (Fig. 4c).

Inoculation with *Bacillus* strains (F = 14.812, P < 0.000), endophyte infection (F = 6.419, P < 0.000), and their interaction (F = 3.245, P = 0.002), all had significant (P < 0.05) effects on root dry weight (Table 3). The root dry weight of EI *A. inebrians* was significantly (P < 0.05) higher than of EF plants under B15 and B96 treatments (Fig. 4d). The root dry weight of B15(EI), B18(EI), B18(EF), B96(EI), B154(EI), B181(EF), B419(EI), and B419(EF) was significantly (P < 0.05) higher than of CK plants, and the root dry weight of B18(EI) was significantly (P < 0.05) higher than B154(EI) and B181(EF) plants (Fig. 4d).

3.5 Membership function value and rank of *A. inebrians* growth parameters

As shown in Table 4, the average membership function value is the average value of 4 germination experiment indicators and 8 pot experiment parameters. The larger the average membership function value is, the better is the comprehensive evaluation of the strain. According to the rank of the average membership function value, three strains were found to have excellent growth promoting ability, specifically B419 > B18 > B181. These three strains showed excellent growth promoting ability both in the seedling stage and the mature stage of the plant, and so could be used in the research of microbial fertilizer. There were three strains with a poorer comprehensive evaluation, namely B106 < B453 < B10. Because the membership function value of their pot experiment parameters was low, the comprehensive evaluation of these three strains was poor.

Table 4 The membership function value and rank of *Bacillus* strains to the growth parameters of *A. inebrians*

Strain number	Membership function value													
	GE	GR	RL1	SL	PH	RL2	TN	SFW	SDW	RFW	RDW	Y	Average value	Rank
B4	0.38	0.00	0.42	0.93	1.00	0.54	0.35	0.16	0.32	0.013	0.313	0.43	0.405	9
B9	0.00	0.69	0.57	0.85	0.97	1.00	0.39	0.15	0.36	0.012	0.346	0.31	0.471	7
B10	0.41	0.43	0.49	0.56	0.08	0.80	0.09	0.15	0.11	0.015	0.104	0.57	0.318	10
B15	0.18	0.18	0.56	0.66	0.05	0.69	0.44	0.31	0.43	0.698	0.445	0.68	0.443	8
B18	0.06	0.75	1.00	0.35	0.84	0.78	0.57	1.00	0.69	1.000	0.711	1.00	0.728	2
B96	0.26	0.68	0.45	0.51	0.48	0.47	0.61	0.70	0.66	0.790	0.671	0.00	0.523	6
B106	0.38	0.69	0.26	0.60	0.00	0.00	0.00	0.00	0.00	0.000	0.000	0.26	0.183	12
B154	1.00	0.49	0.00	0.58	0.72	0.75	0.39	0.58	0.45	0.435	0.456	0.53	0.532	4
B181	0.59	0.69	0.32	0.72	0.72	0.49	0.65	0.43	0.64	0.409	0.631	0.28	0.547	3
B382	0.59	0.56	0.29	0.00	0.57	0.82	0.61	0.70	0.66	0.356	0.652	0.57	0.531	5
B419	0.73	1.00	0.73	1.00	0.71	0.72	1.00	0.77	1.00	0.829	1.000	0.84	0.861	1
B453	0.56	0.69	0.39	0.11	0.30	0.27	0.09	0.10	0.10	0.056	0.095	0.11	0.238	11

Note: GE, GR, RL1, SL, PH, RL2, TN, SFW, SDW, RFW, RDW and Y respectly represent the germination energy, germination rate, radicle length, seeding length, plant height, root length, tiller number, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight and chlorophyll content.

4 Discussion

In order to clarify the effects of *Bacillus* strains, endophyte, and their interaction on seed germination and seedling growth of *A. inebrians*, this study was conducted on EI and EF *A. inebrians* seeds and the subsequent seedlings inoculated with 12 *Bacillus* strains. Four germination indicators (germination energy/rate, radicle and seedling length) and eight pot parameters (plant height, root length, tiller number, chlorophyll concentration, shoot dry/fresh weight, and root dry/fresh weight) were measured. Our results showed that 12 *Bacillus* strains, endophyte and their interaction enhanced seed germination as well as seedling growth of *A. inebrians*, with the B419 strain showing the best growth-promoting ability and so will be used for subsequent studies.

4.1 Effects of endophyte, *Bacillus* and their interaction on seed germination

During the life cycle of higher plants, seed germination and seedling emergence are the most vital and vulnerable stages (Seneviratne et al. 2019). The *Epichloë* endophytes of *A. inebrians* are solely transmitted via asexual vertical seed transmission (Chen et al. 2015), and the presence of the *Epichloë* endophyte can improve the abiotic stress resistance of host plants at the germination stage and initial seeding stages (Zhang et al. 2010b; Chen et al. 2016; Ahmad et al. 2020; Ju et al. 2021). It was also reported that *Epichloë* endophyte infection could enhance the seed germination, seedling length, and radicle length under environmental stress of *Lolium perenne* (Ma et al. 2015), *Elymus tangutorum* (Li et al. 2017), *Roegneria kamoji* (Bu et al. 2018) and *Hordeum brevisubulatum* (Wang et al. 2019). Our results also showed that the presence of *Epichloë* endophyte increased germination energy, seeding length and radicle length of *A. inebrians*. A previous study suggested that the presence of endophyte promoted the seed germination of *A. inebrians* at low temperature (10℃), which might be due to differential expression of fatty acids and alkaloids biosynthesis related genes (Chen et al. 2016).

The seed priming technique using plant growth promoting rhizobacteria (PGPR) has a potential role in stimulating seed germination, vigor, quality and crop yields (Miljakovic et al. 2022; Sohail et al. 2022). Soaking seed with *Bacillus* solution before sowing could effectively promote seed germination (Shahzad et al. 2021; Cardarelli et al. 2022). The treatment of seeds of bitter gourd *(Momordica charantia*) with solutions of some strains of *B. subtilis* has provided good control of damping-off by *Rhizoctonia solani* in *in vitro* studies (Yang and Sung 2011). Our results suggested that 10 strains of 12 *Bacillus* strains could enhance the germination rate of *A. inebrians.* However, the mechanism of *Bacillus* strains promoting seed germination is not clear. The study by Yue et al. (2019) indicated that *B. altitudinis* WR10 increased the germination rate of wheat seeds under salinity stress (200/400 mMNaCl), mostly because of osmolytes secretion, biofilm formation, and by the synthesis of enzymes including catalase, amylase, and ACC deaminase. Meanwhile, it has been proven that volatile organic compounds generated by *Bacillus* strains stimulated seed germination and seedling emergence (Ramirez et al. 2020; Paul et al. 2022). Interestingly, Lastochkin et al. (2020) found that *B. subtilis* 10 − 4 alleviated the passive effect of PEG-6000 seed germination of wheat (*Triticum aestivum*) cv. Ekada70 (drought-tolerant), while worsened this impact under the same state in the case of cv. Salavat Yulaev (drought-sensitive). Li et al. (2021) also observed that *Bacillus* sp. strain X20 isolated from the rhizosphere of wheat inhibited the germination rate, coleoptile length, root length and biomass of wild oat (*Avena fatua*), and had an clearer effect on seed germination than seedling growth. In our study, the treatment with B4 and B15 strains showed insignificant inhibitory effects on the germination rate of *A. inebrians.*

One recent study demonstrated that under different NaCl concentrations, the interaction between *Epichloë* endophyte and PGPR (*Bacillus licheniformis*, *Pseudomonas aeruginosa*, and *Arthrobacter sp.*) enhanced the radical length and the activity of superoxide dismutase of *A. inebrians* seedlings (Ju et al. 2021). In our experiments, the *A. inebrians* was inoculated with 12 *Bacillus* strains and showed that endophyte, *Bacillus* and their interaction enhanced seed germination of *A. inebrians*.

4.2 Effects of endophyte, *Bacillus* and their interaction on seeding growth

Liu et al. (2022) proposed that the shoot and root biomass of EI tall fescue plants were higher than EF plants under saline-alkali stress (200 and 400 mM). When *A. inebrians* plants were exposed to high $CdCl_2$ (100 and 200 μM), endophyte infection increased plant height, tiller number, biomass, activities of antioxidant enzymes and chlorophyll content (Zhang et al. 2010a). The endophyte enhanced plant height, biomass, chlorophyll content, water use efficiency, N and P content of *A. inebrians* under limited water conditions (Xia et al. 2018a). Similar to the previous studies, our results found that the presence of *Epichloë* endophyte promoted the root length, shoot fresh weight, root fresh and dry weight, and chlorophyll content. As explained by Zhong et al. (2021) and Zhao et al. (2021, 2022), the presence of the systemic mutualistic endophyte enhanced the drought resistance of *A. inebrians* plants due to the differential expression of genes related to photosynthesis, sebaceous wax, salicylic and jasmonic acid biosynthesis pathway. Although the presence of *Epichloë* endophytes are a cost to host plants as all of their nutritional requirements come from the plant, a recent study about the exogenous addition of abscisic acid (ABA) showed that ABA might play a role in reducing the negative effects of the endophyte on *A. inebrians* plants that are exposed to drought by preserving growth and enhancing photosynthetic effectiveness (Cui et al. 2022). *A. inebrians* infected by *Epichloë* endophyte showed an increase in content of salicylic acid (SA) hormone concentration and the upregulated expression of genes associated with the SA-signaling pathway, enhancing the plant resistance against the *Blumeria graminis* (Kou et al. 2021).

Numerous *Bacillus* species have been reported as an alternative to chemical fertilizers and pesticides, which could increase the growth and yield of plants (Radhakrishnan et al. 2017). Mendoza-Labrador et al. (2021) demonstrated that *Bacillus* X13, *Bacillus* X14, and co-inoculation of both strains exhibited a promotion effect on total dry biomass, crude protein content and digestibility in *Megathyrsus maximus* under drought. According to Xie et al. (2022), maize root exudate recruited *B. amyloliquefaciens* OR2-30 to colonize the rhizosphere and then inhibited the growth of *Fusarium graminearum* by producing iturins. In our experiments, some *Bacillus* strains increased the growth of *A. inebrians* at the seedling emergence (21 days old) and mature stage (grown in the 2:1 vermiculite and black soil mixture for 3 months). Interestingly, *Bacillus* strain inoculation, except for strain B96, provided a greater increase in growth (root length, tiller numbers, biomass, and chlorphyll content) on the 2–3 month old seedlings than the increase of growth with young seedlings.

Previous studies have shown that organic acids (Mukherjee et al. 2017) and phosphatases (Ambreen et al. 2020) secreted by *Bacillus* could dissolve the inorganic phosphorus in the soil into a plant-available form, thus promoting plant growth. According to Masood et al. (2020), B. *pumilus* produced nitrogenase and had the nifH gene, which fix the nitrogen in the air and supply it to plants to promote plant growth. Yue et al. (2022) suggested that siderophore produced by *Bacillus* sp. WR12 effectively improves the efficiency of iron utilization in soil by wheat. Ayaz et al. (2022) indicated that the *Bacillus* strains NMCN1 and LLCG23 enhanced biomass, plant height and root length of wheat through modulation of phytohormones and salt resistance genes under NaCl stress. It was reported that *Bacillus* spp. secreted phytohormones, such as indole acetic acid (IAA), gibberellins (GAs) and cytokinins (Kang et al. 2019; Ji et al. 2020; Jiao et al. 2022). Citric acid in rice root exudates recruited *B. altitudinis* LZP02 to colonize the roots, thereby promoting plant growth, and *B. altitudinis* LZP02 increased the secretion of citric acid (Jiao et al. 2022). The chlorophyll is the necessary pigment for plants to harvest light energy (Simkin et al. 2022). There is a close relationship between photosynthetic capacity and plant biomass production (Yang et al. 2014). Zhang et al (2019) found that inoculation of *B. pumilus* reduced the damage of chloroplast structure caused by drought, increase the chlorophyll content and photosynthesis rate, thus increase the biomass of *Glycyrrhiza uralensis*.

5 Conclusion

In conclusion, the present research demonstrated that *Epichloë* endophyte, *Bacillus* strains, and their interaction showed important roles in promoting seed germination and plant growth, as reflected by four germination indicators and eight growth parameters. Moreover, the B419 strain had excellent growth promotion ability as confirmed by membership function analysis. The B419 strain will be further investigated to obtain understanding of the mechanism of conferred resistance of host plants against biotic and abiotic stresses.

Declarations

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Competing Interests

The authors declare no conflict of interest.

Author Contributions

XXZ and YZL designed the experiments. *Bacillus* strains were provided by XMN. HTZ performed the experiments, analyzed the data and wrote the manuscript. MJC, XXZ, YZL, YWJ, WZ, and HTZ revised the manuscript.

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Figures

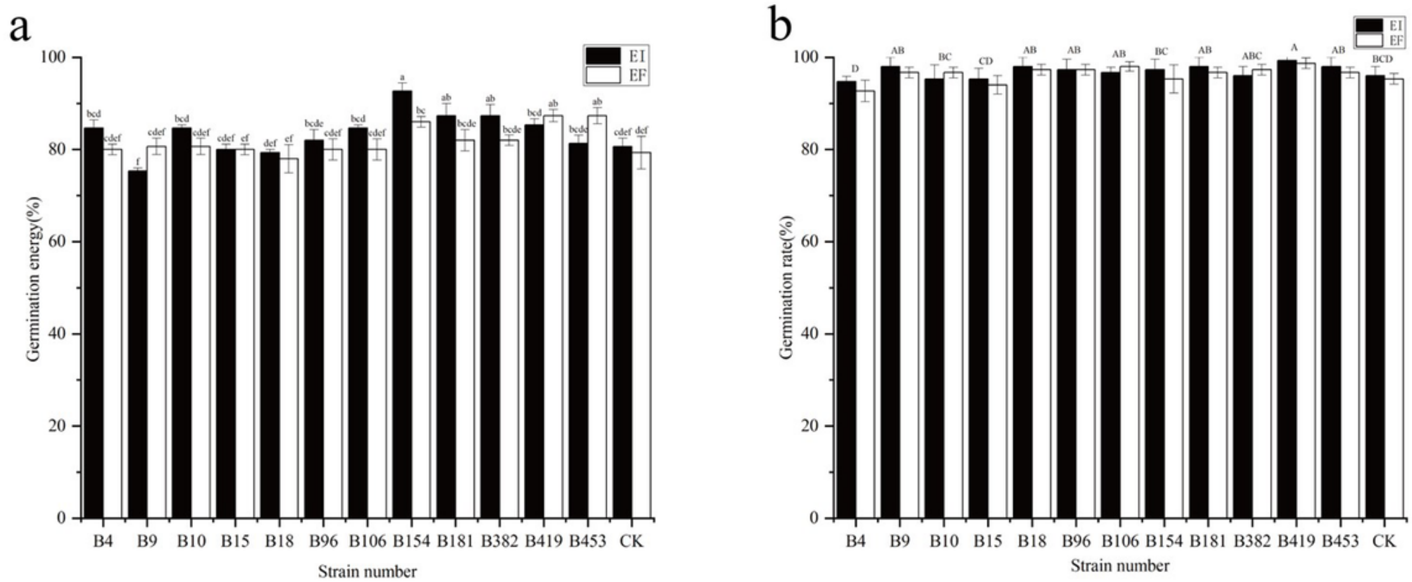

Figure 1

Effects of *Epichloë*endophyte and *Bacillus* strains inoculation on germination energy (a) and rate (b) of *A. inebrians*. Different capital letters indicate significant (P<0.05) differences among different strains at 0.05 level. Different small letters indicate significant (P<0.05) differences among the intercation between all treatments.

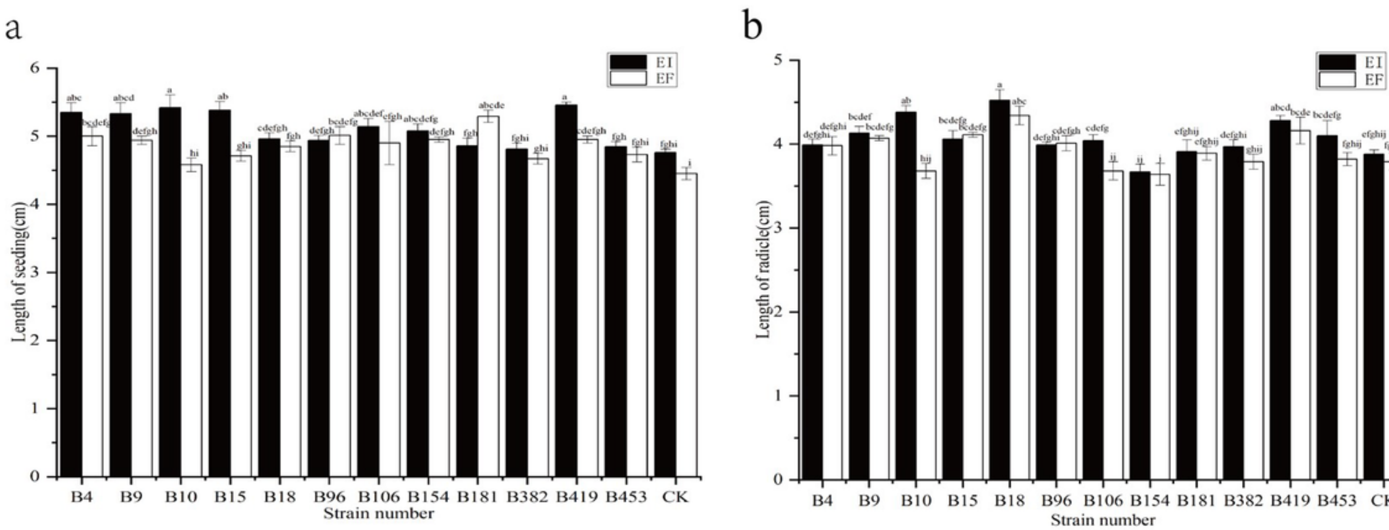

Figure 2

Effects of *Epichloë* endophyte and *Bacillus* strains inoculation on seeding length (a) and radicle length (b) of *A. inebrians*. Different small letters indicate significant (P<0.05) differences among all treatments.

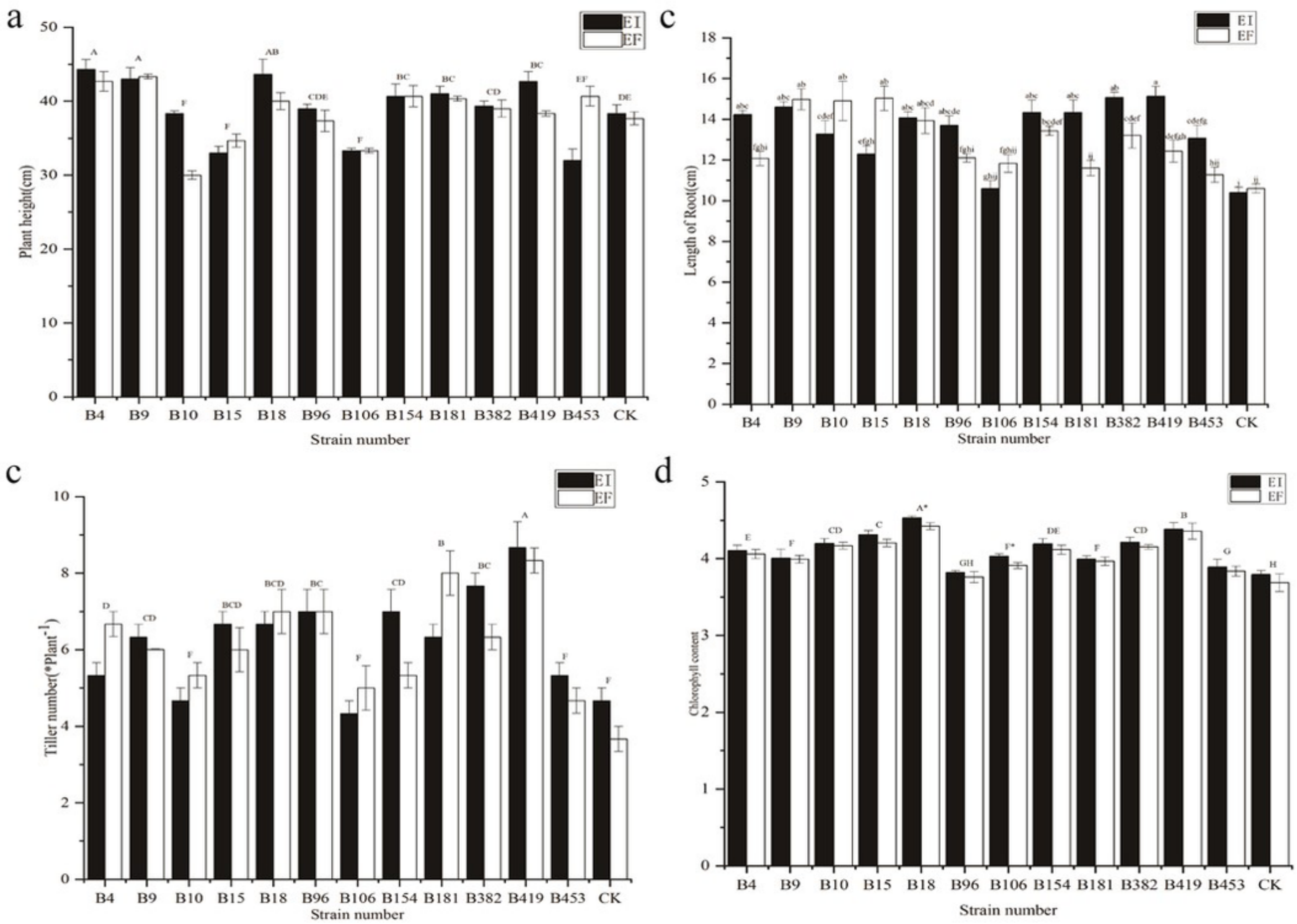

Figure 3

Effects of *Epichloë* endophyte and *Bacillus* strains inoculation on plant height (a), root length (b), tiller number (c) and Chlorphyll content (d) of *A. inebrians*. The asterisk (*) means significant difference at $P<0.05$ (independent t-test) between endophyte-infected (EI) and endophyte-free (EF) plant at corresponding different *Bacillus* strains at 0.05 level. Different capital letters indicate significant ($P<0.05$) differences among different strains at 0.05 level. Different small letters indicate significant ($P<0.05$) differences among all treatments.

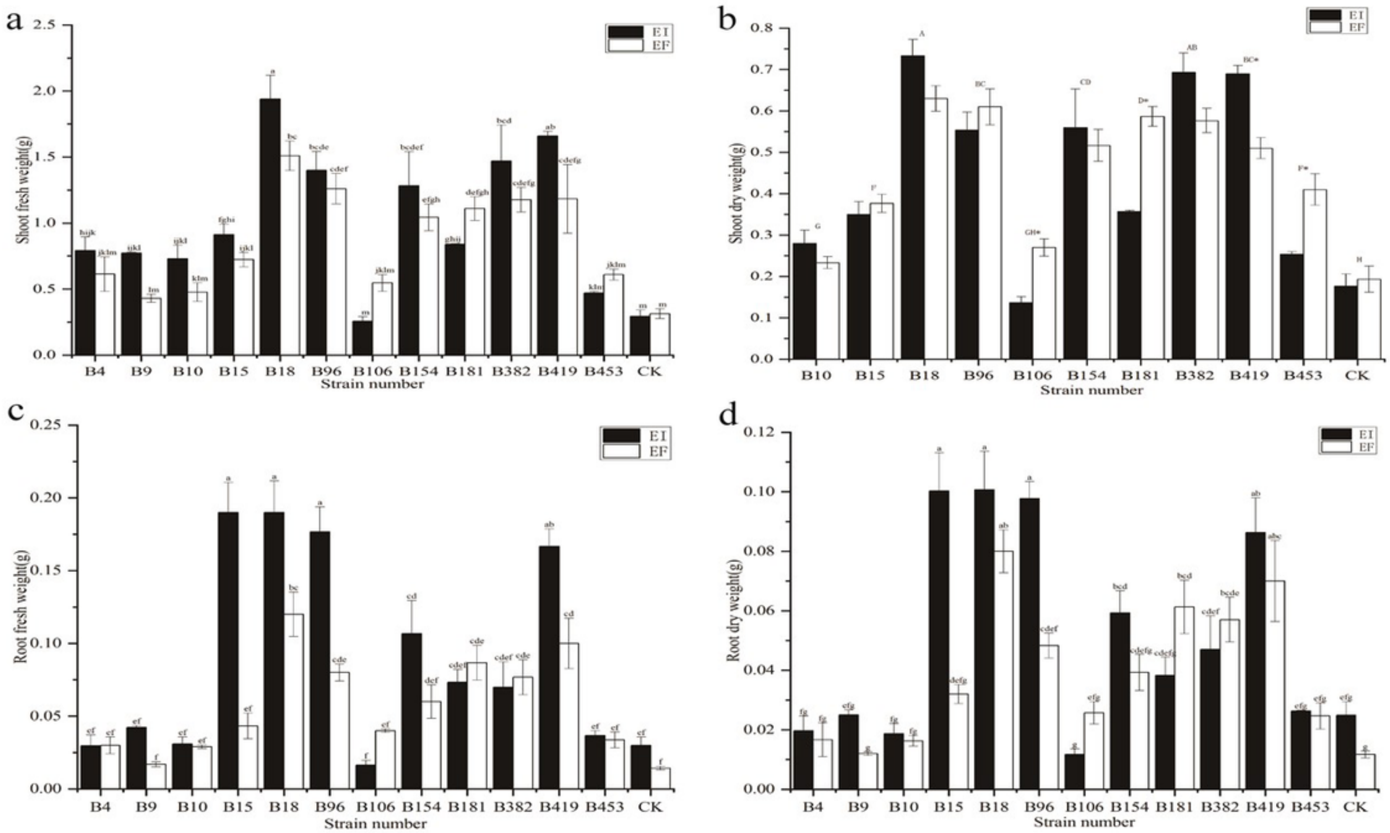

Figure 4

Effects of *Epichloë* endophyte and *Bacillus* strains inoculation on shoot fresh weight (a), shoot dry weight (b), root fresh weight (c) and root dry weight (d) of *A. inebrians*. The asterisk (*) means significant difference at P<0.05 (independent t-test) between endophyte-infected (EI) and endophyte-free (EF) plant seeds at corresponding different *Bacillus* strains at 0.05 level. Different capital letters indicate significant (P<0.05) differences among different strains at 0.05 level. Different small letters indicate significant (P<0.05) differences among all treatment.