

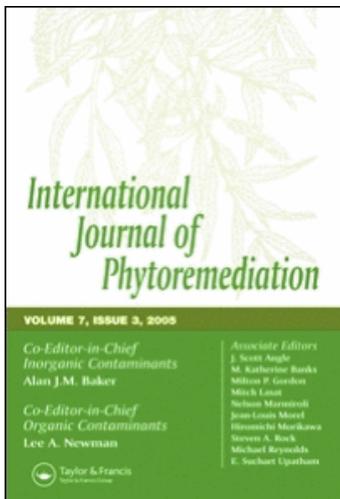
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SEXUAL PROPAGATION OF *PTERIS VITTATA* L. INFLUENCED BY PH, CALCIUM, AND TEMPERATURE

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We aimed to optimize germination and growth conditions of the arsenic hyperaccumulating fern, Pteris vittata L. Pot experiments were carried out to investigate the effects of soil pH, soil calcium (Ca) concentration, and temperature on the sexual propagation of P. vittata. At 25°C, germination was both accelerated and increased by high soil pH and Ca concentration. Spores of P. vittata did not germinate on medium with a pH of 4.6. Amending strongly acid soils with 27.5 or 40 μmol/g Ca(OH)₂ significantly improved the growth rate during both the germination phase and the gametophyte phase. Amending strongly acid soils with NaOH (55 μmol/g) promoted germination, but did not affect subsequent growth. Among the different temperature, germination and growth rates were higher at 25°C than at 20°C or 30°C. The distribution of P. vittata in China might be influenced by its requirement for high pH and high Ca concentration in the soil, and appropriate growth temperature to complete sexual propagation. These results provided important information for improving breeding conditions of P. vittata and will be helpful for extending the range of areas in which P. vittata can be used for phytoremediation.

KEYWORDS Arsenic hyperaccumulator, calcium, gametophyte, germination, soil

INTRODUCTION

The fern species *Pteris vittata* L. is an efficient phytoremediator, because it can accumulate large amounts of arsenic, and it has a large biomass (Chen *et al.*, 2002a; Tu *et al.*, 2004; Wang *et al.*, 2006). There have been several successful field experiments utilizing *P. vittata* to remove arsenic from contaminated soils (Liao *et al.*, 2004; Kertulis-Tartar *et al.*, 2006). For use in large-scale phytoremediation, it is essential to obtain sufficient *P. vittata* sporelings rapidly and inexpensively.

P. vittata is an indicator plant for calcareous soil (ECF, 1990; SCBG, 1964). According to surveys of *P. vittata* throughout 20 provinces in China (Chen *et al.*, 2005), nearly 50% grow on calcareous soils. At the same time, they are distributed mainly in tropical or

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semi-tropical regions (Chen *et al.*, 2005). Therefore, *P. vittata* may have some special requirements for soil pH, Ca concentration and temperature.

As in all ferns, *P. vittata* has alternating haploid gametophyte and diploid sporophyte generations. The diploid sporophytic generation develops from the zygote, the fusion product of haploid gametes, including the male antheridia and female archegonia. Sporophyte cells undergo meiosis to produce haploid spores, which divide mitotically to form the multicellular gametophyte, which subsequently differentiate into gametes (Traverse, 2007). Germination is regarded as the first phase in the sexual propagation of ferns. Therefore, the distribution of *P. vittata* is limited to areas where spores can germinate. Another important factor affecting the natural distribution of *P. vittata* is the growing conditions of the gametophyte, since the haploid gametophyte generation is the most vulnerable phase throughout ferns' sexual propagation cycle (Christopher, 2002).

In this study, we investigated the effects of soil pH, Ca concentration, and temperature on the germination and growth rate of *P. vittata* gametophyte and sporophyte. We aimed to understand how these environmental factors influence the natural distribution of *P. vittata* and how optimizing these variables could improve the efficiency of *P. vittata* propagation. This information is useful for developing wider phytoremediation applications of *P. vittata*.

MATERIALS AND METHODS

Soils and Plant Culture

Semi-Luvisols from Beijing and Ferralsols from Hunan province were collected for pot experiments. The properties of Semi-Luvisols and Ferralsols are shown in Table 1. The two soils differed markedly from each other in pH and Ca concentration.

Table 1 Properties of semi-luvisols and ferralsols

Soil type	pH	g/kg				%	
		Organic matter	Total P	Total N	CEC	Ca	Clay content
Semi-luvisols	8.0	18.5	0.88	1.04	14.2	6.88	26.0
Ferralsols	4.6	26.7	0.33	1.82	11.8	0.08	39.7

Fertile fronds of *P. vittata* were collected and air-dried. Sporangia were gently scraped from pinnae and then mixed thoroughly with deionized water. The spore suspension was centrifuged at $600 \times g$ for 5 min. Empty sporangia floating on the top were discarded. The pelletized spores were re-suspended in deionized water then sown on soil at a density of 10 per cm^2 . Each treatment had 10 replicates. The growing conditions during the experiment were as follows: 16 h light, 8 h darkness; relative humidity, 70%; and temperature, 25°C. The same conditions were used in the temperature-dependent experiments, except that temperature was adjusted to 20, 25, or 30°C.

EXPERIMENTAL DESIGN

Experiment 1: Effects of pH and Ca. Spores of *P. vittata* were sown on six kinds of media, which were prepared by mixing Semi-Luvisols (S) and Ferralsols (F) at different ratios (S:F = 1:0, 2:1, 1:1, 1:2, 1:15, 0:1). The pH and Ca concentrations are shown in Table 2 (Chen *et al.*, 2002b).

Table 2 Growth rates of *P. vittata* in mixed soils

S:F (w/w)	pH	Ca (%)	PG (%)	GAT (d)	GFT (d)	PS (%)	SAT (d)	SFT (d)	2 cm time (d)
1:0	8.0	6.88	40.0 ± 1.9 a	14 ± 0 a	49 ± 1 a	42.4 ± 1.1 a	66 ± 1 a	70 ± 3 a	136 ± 3 a
2:1	7.5	4.61	34.1 ± 1.9 b	15 ± 0 b	49 ± 1 a	58.9 ± 1.3 b	66 ± 1 a	61 ± 2 b	127 ± 2 b
1:1	7.3	3.48	33.0 ± 1.8 b	15 ± 0 b	47 ± 1 a	69.4 ± 2.0 c	65 ± 1 a	58 ± 2 b	123 ± 2 b
1:2	7.0	2.35	24.9 ± 1.5 c	17 ± 0 c	48 ± 2 a	62.8 ± 1.4 b	70 ± 2 b	44 ± 1 c	115 ± 2 c
1:15	6.3	0.47	21.9 ± 1.3 c	19 ± 0 d	75 ± 1 b	81.7 ± 2.1 d	99 ± 1 c	22 ± 1 d	121 ± 1 b
0:1	4.6	0.08	0	—	—	0	—	—	—

Data are means ± standard deviation. Means followed by different letters in a column are significantly different at $p < 0.05$ ($n = 10$). S:F indicates ratio of Semi-Luvisols soil to Ferralsols soil. Spores on treatment S:F = 0:1 did not germinate during the 180-day incubation period. PG, GAT, GFT, PS, SAT and SFT represent Percentage Germination, Gametophyte Appearance Time, Gametophyte Formation Time, Percentage of Sporophyte, Sporophyte Appearance Time, and Sporophyte Formation Time, respectively.

Experiment 2: Effects of $\text{Ca}(\text{OH})_2$, NaOH and CaCl_2 . Spores of *P. vittata* were sown on Ferralsols soils with various amendments (Table 3) as follows: (1) addition of 27.5 $\mu\text{mol/g}$ $\text{Ca}(\text{OH})_2$, which increased soil pH to 6.5, hereafter referred to as “low $\text{Ca}(\text{OH})_2$ ”; (2) addition of 40 $\mu\text{mol/g}$ $\text{Ca}(\text{OH})_2$, which increased soil pH to 7.3, hereafter “high $\text{Ca}(\text{OH})_2$ ”; (3) addition of 55 $\mu\text{mol/g}$ NaOH, which increased soil pH to 6.6, hereafter “low NaOH”; (4) addition of 80 $\mu\text{mol/g}$ NaOH, which increased soil pH to 7.3, hereafter “high NaOH”; (5) addition of 27.5 $\mu\text{mol/g}$ CaCl_2 , which increased soil Ca concentration to the same level that in the low $\text{Ca}(\text{OH})_2$ treatment, hereafter “low CaCl_2 ”; and (6) addition of 40 $\mu\text{mol/g}$ CaCl_2 , which increased soil Ca concentration to the same level as that in the high $\text{Ca}(\text{OH})_2$ treatment, hereafter “high CaCl_2 ”. No amendments were added to the control.

Table 3 Growth parameters during early stages of *P. vittata* lifecycle in Ferralsols with different amendments

Treatment	pH	PG (%)	GAT (d)	GFT (d)	PS (%)
Ca (OH) ₂ , 27.5 $\mu\text{mol/g}$	6.5	28.6 ± 2.7 a	28 ± 1 a	98 ± 9 a	73.3 ± 9.1 a
Ca (OH) ₂ , 40 $\mu\text{mol/g}$	7.3	39.9 ± 2.7 b	24 ± 0 b	115 ± 2 b	36.7 ± 12.8 b
NaOH, 55 $\mu\text{mol/g}$	6.6	24.6 ± 3.9 a	35 ± 1 c	—	—
NaOH, 80 $\mu\text{mol/g}$	7.3	—	—	—	—
CaCl ₂ , 27.5 $\mu\text{mol/g}$	4.2	—	—	—	—
CaCl ₂ , 40 $\mu\text{mol/g}$	4.1	—	—	—	—
Control (CK)	4.6	—	—	—	—

Data are means ± standard deviation. Means followed by different letters in a column are significantly different at $p < 0.05$ ($n = 10$). Germination did not occur in treatment with higher NaOH and in both CaCl_2 treatments. PG, GAT, GFT, and PS represent Percentage Germination, Gametophyte Appearance Time, Gametophyte Formation Time, and Percentage of Sporophyte, respectively.

Experiment 3: Effects of incubation temperature. *P. vittata* was grown at 20, 25, and 30°C. Other growth conditions were the same as the S:F = 1:0 treatment in Experiment 1.

Growth Indexes

Ten days after sowing, digital photos were taken every day to record *P. vittata* Germination. We considered that all the fertile spores had germinated when no further

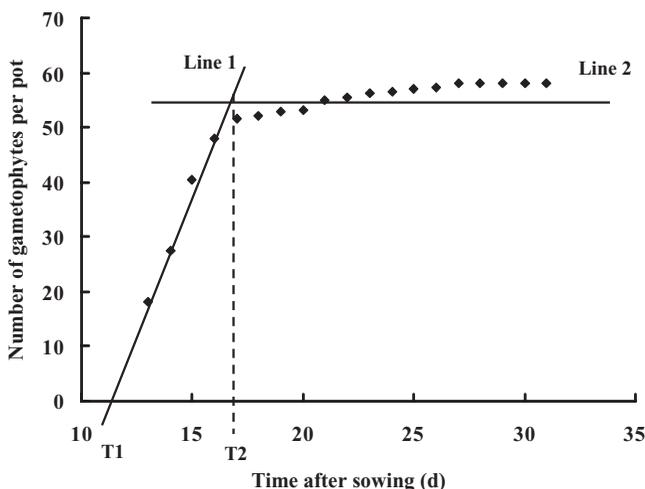


Figure 1 Typical germination curve of *P. vittata*. Each point represents mean number of gametophytes ($n = 10$).

emergence of gametophyte was observed. The *Percentage Germination (PG)* is defined as follows:

$$PG (\%) = Ngp/N_{spore}$$

Where Ngp is the number of gametophytes per pot, and N_{spore} is the number of spores sown per pot. Figure 1 illustrates derivation of the *Gametophyte Appearance Time (GAT)*. A straight line (Line 1) is fitted on the initial arithmetically increasing phase of gametophyte emergence and another line (Line 2) is fitted on the plateau phase when no further emergence is detected. The intersection point of Line 1 and the abscissa is defined as the germination starting Time (T_1). The intersection point of Line 1 and Line 2 is defined as the germination finishing Time (T_2). The mean of T_1 and T_2 is defined as the *GAT*:

$$GAT (d) = (T_1 + T_2)/2$$

The numbers of sporophytes were recorded everyday after the first sporophyte appeared. At the same time, 10 plants in each treatment in the temperature experiment were randomly chosen to record the *Gametophyte Diameter (GD)*, the longest equatorial diameter of the cordate-thalloid gametophyte. *Percentage of Sporophytes (PS)* and *Sporophyte Appearance Time (SAT)* were defined in the same way as *PG* and *GAT*, respectively. The time required by each gametophyte to produce a sporophyte is defined as *Gametophyte Formation Time (GFT)*:

$$\begin{aligned} PS (\%) &= N_{sp}/Ngp, \\ SAT (d) &= (bSAT + eSAT)/2, \text{ and} \\ GFT(d) &= SAT - GAT, \end{aligned}$$

where N_{sp} is defined as the number of sporophytes per pot. The time that the first sporophyte appeared is designated as $bSAT$ (d); and the time that the last sporophyte

appeared as *eSAT* (d). When no more sporophyte appeared, five were randomly chosen to remain in each treatment, while the others were removed to retain the same sporophyte density in each treatment. The time taken by each sporophyte to reach 2 cm in height is designated as the *Sporophyte Formation Time (SFT)*. The total time required by a spore to become a 2-cm-high sporophyte is defined as the *2 cm Time*:

$$2 \text{ cm Time (d)} = \text{SFT} + \text{SAT}.$$

Chemical Analysis and Statistical Methods

Soils were air-dried and ground. Soil pH was measured in a 1:2.5 (w/v) soil–water solution. The soils were digested using $\text{HNO}_3\text{-H}_2\text{O}_2$ (USEPA, 1996), and then Ca concentrations were determined by flame atomic absorption spectrometry (Vario 6, Jena Co. Ltd., Germany). Standard reference materials for soil (GBW-07401) obtained from the China National Center for Standard Reference Materials were digested along with the samples to ensure accuracy. Data were statistically analyzed using SPSS 13.0 (SPSS Inc., Chicago, U.S.A.). Data were compared using ANOVA.

RESULTS

Effects of Soil pH and Ca Concentration on Germination and Growth of *P. vittata*

Higher soil pH and Ca concentration facilitated germination and the appearance of *P. vittata* sporophyte, but did not accelerate sporophyte growth (Table 2). Spores on Ferralsols soil with pH 4.6 and 0.08% Ca did not germinate during the 180-day incubation period, while germination in other treatments occurred 14–19 days after sowing (Table 2).

Germination of *P. vittata* gametophyte decreased as the Semi-Luvisols:Ferralsols ratio decreased (Figure 2). *PG* was positively correlated with both soil pH and Ca concentration (Table 4). On S:F = 1:0 soil (pH 8.0 and 6.88% Ca), germination was significantly higher than in the other five treatments ($p < 0.05$), and was 77.8% higher than the S:F = 1:15 treatment (Table 2). Gametophyte appearance was delayed as the Semi-Luvisols:Ferralsols ratio decreased. *GAT* was negatively correlated with both soil pH and Ca concentration (Table 4). On S:F = 1:0 soil, *P. vittata* gametophytes appeared approximately 14 days after sowing, 5 days earlier than in the S:F = 1:15 treatment. There were no significant differences in *GFT* among the treatments, except in the S:F = 1:15 treatment, in which *GFT* was almost 60% longer than in other treatments (Table 2).

The *PS* increased as the Semi-Luvisols:Ferralsols ratio decreased (Table 2). *PS* was negatively correlated with both soil pH and Ca concentration (Table 4). The S:F = 1:15 treatment had the highest *PS*, which was 30% higher than that of the S:F = 1:2 treatment (Table 2). With respect to *SAT*, differences between treatments were not statistically significant at $p < 0.05$ except the S:F = 1:15 treatment, which had a longer *SAT* than in other treatments. *SAT* was 28 days later in S:F = 1:15 than in S:F = 1:2. Noticeably, although the S:F = 1:15 treatment had the highest *PS*, the *SAT* of this treatment was the longest. The particularly long period required for sporophyte growth in the S:F = 1:15 treatment may be due to the late *GAT* (19 days) coupled with the long *GFT* (75 days). *SAT* was significantly correlated with *GAT* at $p < 0.05$, and *GFT* at $p < 0.01$ (Table 4).

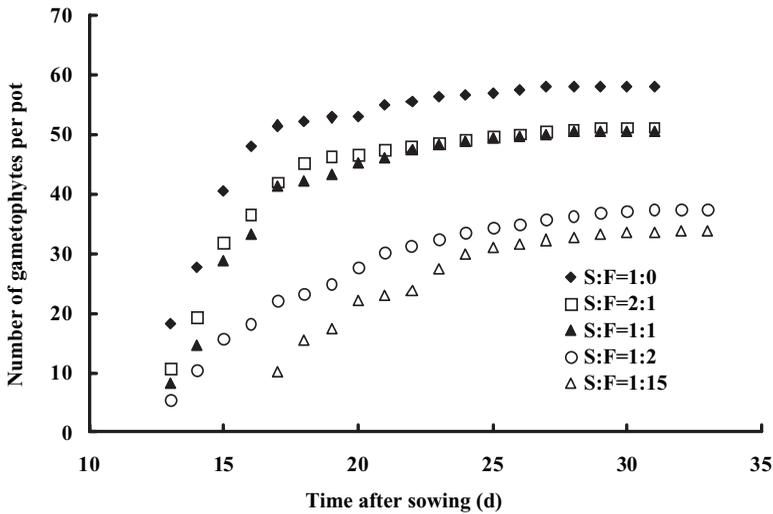


Figure 2 Germination curves of *P. vittata* on different soils. Each point represents mean number of gametophytes ($n = 10$).

The *SFT* reduced with decreasing Semi-Luvisols:Ferralsols ratio (Table 2), and was positively correlated with both soil pH and Ca concentration (Table 4). There was significant differences among the six treatments, except between treatments S:F = 2:1 and S:F = 1:1 ($p < 0.05$). *SFT* of the S:F = 1:15 treatment was much shorter than that of other treatments, and was only 31.4% of the treatment S:F = 1:0. As the Semi-Luvisols:Ferralsols ratio increased, the *2cm Time* decreased from 136 days to 121 days (Table 2). This trend was similar to that of *SFT*, but had a lower magnitude (Table 4).

Table 4 Correlation coefficients between soil properties and growth indices of *P. vittata* on mixed soils

	Correlation coefficients							
	Ca (%)	PG (%)	GAT (d)	GFT (d)	PS (%)	SAT (d)	SFT (d)	2 cm time (d)
pH	0.99**	0.95*	-0.94*	-0.76	-0.95*	-0.83	0.97**	0.77
Ca (%)	-	0.97**	-0.92*	-0.69	-0.94*	-0.76	0.95*	0.84
PG (%)		-	-0.94*	-0.66	-0.84	-0.76	0.95*	0.86
GAT (d)			-	0.85	0.81	0.92*	-0.99**	-0.65
GFT (d)				-	0.68	0.99**	-0.85	-0.20
PS (%)					-	0.72	-0.86	-0.71
SAT (d)						-	-0.92*	-0.33
SFT (d)							-	0.68
2 cm time (d)								-

*Indicates significant correlation at $p < 0.05$ ($n = 10$);

**Indicates significant correlation at $p < 0.01$ ($n = 10$). PG, GAT, GFT, PS, SAT and SFT represent Percentage Germination, Gametophyte Appearance Time, Gametophyte Formation Time, Percentage of Sporophyte, Sporophyte Appearance Time and Sporophyte Formation Time, respectively.

Effects of Amendments on Germination and Subsequent Growth of *P. vittata*

$\text{Ca}(\text{OH})_2$ improved both germination and the subsequent growth of *P. vittata*. The germination rate increased as the amount of $\text{Ca}(\text{OH})_2$ increased, but sporophyte emergence was not affected. NaOH at $55 \mu\text{mol/g}$ improved germination but did not affect emergence of sporophytes. CaCl_2 did not affect either gametophytes or sporophytes.

No spores germinated on Ferralsols soil without any amendment (control). Germination occurred on the low $\text{Ca}(\text{OH})_2$ and high $\text{Ca}(\text{OH})_2$ treatments 28 and 23 days after sowing, respectively. The *PG* of the high $\text{Ca}(\text{OH})_2$ treatment was 20% higher than that of the low $\text{Ca}(\text{OH})_2$ treatment (Table 3). On the contrary, the *GFT* of the high $\text{Ca}(\text{OH})_2$ treatment was nearly 18 days longer than that of the low $\text{Ca}(\text{OH})_2$ treatment, and the *PS* of the high $\text{Ca}(\text{OH})_2$ treatment was only 50% of that of the low $\text{Ca}(\text{OH})_2$ treatment. Gametophytes appeared on the low NaOH treatment 35 days after sowing (Table 3). No germination occurred on the high NaOH , high CaCl_2 , or low CaCl_2 treatment during the 180-day incubation period (Table 3). Sporophytes appeared on the low $\text{Ca}(\text{OH})_2$ and high $\text{Ca}(\text{OH})_2$ treatments 126 and 138 days after sowing, respectively (Table 3). No sporophytes emerged on the low NaOH treatment during the 180-day incubation period.

Compared with *P. vittata* on the various Semi-Luvisols:Ferralsols soils, *P. vittata* on soils with amendments grew more slowly (Tables 2 and 3). Both *PG* and *PS* were lower while both *GAT* and *GFT* were longer on soils with added chemicals. None of the sporelings on amended soils reached 2 cm in height during the 180-day incubation period (Table 3).

Effects of Temperature on Germination and Growth of *P. vittata*

Temperature had marked effects on the growth of *P. vittata*. A higher spore germination rate was observed at 25°C , compared with that at 20°C or 30°C (Figure 3). The *PGs* of these three treatments were ranked as follows: $25^\circ\text{C} > 20^\circ\text{C} > 30^\circ\text{C}$, being 40.0, 28.5, and 14.9% respectively, while *GAT* was $25^\circ\text{C} < 30^\circ\text{C} < 20^\circ\text{C}$, being 14, 25 and 28 d, respectively (Table 5). Interestingly, *GAT* at 30°C was earlier than that at 20°C , but *PG* was lower at 30°C .

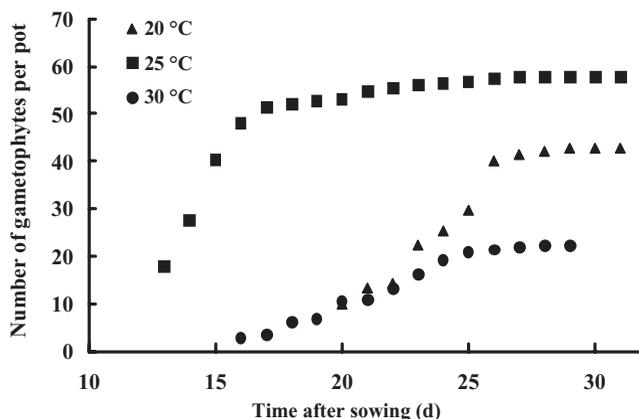


Figure 3 Germination curves of *P. vittata* at different temperatures. Each point represents mean number of gametophytes ($n = 10$).

Table 5 Growth rates of *P. vittata* at different temperatures

Temperature (°C)	PG (%)	GAT (d)	GD (cm)	GFT (d)	PS (%)	SFT (d)
20	28.5 ± 2.4 a	28 ± 2 a	0.22 ± 0.01 a	139 ± 2 a	18.5 ± 2.3 a	—
25	40.0 ± 1.9 b	14 ± 0 b	0.43 ± 0.04 b	49 ± 1 b	42.4 ± 1.1 b	70 ± 3 a
30	14.9 ± 1.5 c	25 ± 1 c	0.11 ± 0.01 c	—	—	—

Data are means ± standard deviation. Means followed by different letters in a column are significantly different at $p < 0.05$ ($n = 10$). No sporophyte was observed during the 180-day incubation at 30°C. PG, GAT, GD, GFT, PS, and SFT represent Percentage Germination, Gametophyte Appearance Time, Gametophyte Diameter, Gametophyte Formation Time, Percentage of Sporophyte, and Sporophyte Formation Time, respectively.

At 30°C, no sporophyte appeared during the 180-day incubation period, while at 20°C, sporophyte appeared 167 days after sowing (GAT+GFT, Table 5). The mean diameters of gametophyte grown at both 20°C and 30°C were smaller than that of gametophytes grown at 25°C (Table 5). Sporophytes grown at 20°C failed to reach 2 cm during the 180-day incubation period. At 25°C, sporophytes appeared 63 days after sowing (GAT + GFT) and reached 2 cm in height 70 days after emergence (Table 5).

DISCUSSION

In this study, we found that soil pH, Ca concentration, and growth temperature had significant impacts on the germination of *P. vittata* and its subsequent growth.

Impacts of Soil pH and Ca Concentration on *P. vittata* Germination

In previous reports, it was suggested that the distribution of *P. vittata* was concentrated around limestone (SCBG, 1964; ECF, 1990). Scheuerlein *et al.* (1989) indicated that the Ca^{2+} influx from the medium into the spores might be important in phytochrome-mediated germination of spores of *Dryopteris paleacea* Sw. Wayne and Hepler (1984) reported that calcium was required for spore germination of *Onoclea sensibilis* L., a phytochrome-mediated fern, and that it may be acting as a second messenger. In the present study, we also observed that *P. vittata* spores required calcium for successful germination.

With respect to pH, many previous studies had reported that spores of most fern species germinated at slightly acidic or neutral pH (Raghavan, 1989). However, spores of some fern species, including *Notholaena cochisensis* and *Pellaea limitanea*, germinated on soils with pH as high as 9–10 (Hevly, 1963). The highest germination of the fern *Drynaria fortunei* occurred at weak alkalinity (pH = 7.7) (Chang *et al.*, 2007). George *et al.* (2007) concluded that in culture media, the detrimental effects of an adverse pH were generally related to ion availability and nutrient uptake, rather than cell damage. Wayne *et al.* (1986) reported that increasing the medium's pH from 5.8 to 7.2 did not affect the germination of fern *Onoclea sensibilis* L. In the present study, we found that soil pH affected the germination of *P. vittata*, and that higher pH was better for germination.

Reasons for the Restricted Natural Distribution of *P. vittata* in China

P. vittata is mainly distributed on alkaline calcareous soils in semitropical or tropical regions (SCBG, 1964; ECF, 1990), where the environmental condition satisfy all the

germination requirement of *P. vittata* spores, *i.e.*, high pH and high Ca concentration (Tables 2, 3). Therefore, the natural distribution of *P. vittata* might be limited to areas with both high pH and high Ca concentration, where it can complete its natural sexual propagation cycle.

Temperature also plays an important role in determining the distribution of *P. vittata* in China. *P. vittata* mainly grows in tropical and subtropical regions to the south of Qin Mountain or in areas where temperature exceeds 0°C in January (ECF, 1990; Chen *et al.*, 2005). The results of the present study indicated that *P. vittata* could germinate at 20°C to 30°C, but the *PG* was higher and the *GAT* was shorter at 25°C, (Figure 3). In July, the average temperature in the southernmost province of China is less than 30°C, which demonstrates that the highest temperatures in southern China do not threaten growth of *P. vittata*. Therefore, it can be inferred here that low temperature is a more important factor restricting the distribution of *P. vittata* to the south of Qin Mountain, considering that the average annual temperature is lower than 20°C in most areas north of Qin Mountain.

In a previous study, germination of the fern *Schizaea pusilla* was enhanced by raising the temperature to 25°C (Lu *et al.*, 2002). However, when the temperature was raised to 32°C, germination was inhibited because the high temperature impaired essential phytochrome-mediated process within the cells (Ranal, 1999). *P. vittata* had a short *GAT* but a low *PG* at 30°C. In addition, the gametophyte fertilization rate was lower at 30°C, and the gametophyte diameter was smaller (Table 5). Niu *et al.* (2002) suggested that fertilization of gametophyte with very small diameter was difficult. Therefore, it is likely that the decreased germination at 30°C results from impaired cell activity, and that the slow maturity rate of gametophyte results from their small diameter.

Possible Measures to Widen the Range of *P. vittata* for Phytoremediation

Adding various substances to soil, *e.g.*, Ca(OH)₂, could widen the areas in which *P. vittata* could be used for phytoremediation of arsenic-contaminated soils. Simultaneously increasing soil pH and calcium concentration by adding Ca(OH)₂ accelerated germination and the appearance of the sporophyte, but did not affect sporophyte growth (Table 3). Increasing pH alone also enhanced the germination, but did not facilitate the appearance of sporophyte (Table 3) while increasing calcium concentration alone did not improve germination. We can conclude that increasing both soil pH and Ca concentration can facilitate the germination and the appearance of sporophyte on acid soils with low Ca concentration. An appropriate temperature is also an important factor for improving the speed and efficiency of *P. vittata* reproduction. In our study, spores showed the highest germination rate at 25°C. Thus, spore-breeding of *P. vittata* should be carried out when climatic conditions are close to this ideal temperature, or could be conducted in temperature-controlled greenhouses in areas where *P. vittata* is required to remediate contaminated soils.

In conclusion, our results show that soil pH and Ca concentration significantly influenced the germination and the subsequent growth of *P. vittata*. *P. vittata* could not germinate in strong acid soils (pH = 4.6). Higher Ca and higher pH improved germination of gametophyte and sporophyte. In contrast, lower pH and lower Ca facilitated sporophyte growth. Addition of Ca(OH)₂ effectively enhanced germination and growth of *P. vittata* gametophyte, whereas addition of CaCl₂ and NaOH had no clear effects. The optimal temperature for germination and development of *P. vittata* was 25°C.

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