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Effect of hormones on the seed germination of *Bupleurum* species

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In Asia, *Radix Bupleuri* (*Bupleurum* spp. root) is an important medicine used for treating many diseases over the past 2000 years. However, its cultivation is difficult due to poor germination rates and loss of seedling viability shortly after seeding. In the present study, the effect of 6-benzyl aminopurine (6-BA), daminozide, gibberellin (GA3), paclobutrazol, uniconazole, and mepiquat chloride (MC) on the germination of seeds of 'Zhongchai No. 3', which is the newest released commercial variety of *Bupleurum chinense* DC in China was analyzed. Only GA3 was found to promote seed germination significantly, and its optimum concentration was 223.84 to 238.78 mg·L^{-1}. In addition, incubation with GA3 on the first day of germination was extremely important for seedling survival. This protocol could also be applied to 'Zhonghongchai No. 1' (*B. scorzonerifolium* Willd.) and 'Hubei chaihu' (*B. chinense* DC.). The combination of GA3 treatment and centralized incubation could be an efficient way to industrialize the production of secondary metabolites from *Bupleurum* species.

Key words: Hormone, *Bupleurum* spp., germination, gibberellin (GA3).

INTRODUCTION

Radix Bupleuri (root of *Bupleurum* spp.) is one of the most important medicinal herbs in Eurasia and North Africa for treating fever, chronic hepatitis, kidney syndrome, inflammatory diseases, menstrual disorder, and digestive system ulcers (Pistelli et al., 1996; Guo et al., 2000; Ikegami et al., 2006; Mabberleyl, 2008). Utilization of *Bupleurum* species in preparations was firstly recorded for more than 2000 years in Shen-Nong's Herbal of China (Xie et al., 2009).

Nearly 250 bioactive compounds from this genus have been identified through phytochemical investigations; in particular, saikosaponin *a* and saikosaponin *d* are known for their pharmacological activities (Ashour and Wink, 2011). *Bupleurum* species are officially listed in the Chinese and Japanese Pharmacopoeias in addition to the WHO monographs of the commonly used medicinal plants of China and Korea (WHO, 1997, 1998). Due to its medicinal importance, the demand for *R. Bupleuri* has increased steadily in recent years. Nearly eight million kilograms of *R. Bupleuri* are required each year in China, and gross sales of manufactured prescription medicines containing

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R. Bupleuri in Japan amounted to 27 billion yen in 2002 (Pan, 2006; Zhu et al., 2009).

Due to the annual increased market requirement and considerable exploitation of *Bupleurum* species, natural wild resources have decreased sharply. Wild *R. Bupleuri* shows high variability in its pharmacological active components. Therefore, the cultivated *Bupleurum* has become economically important. In the last 10 years, three commercial varieties (Zhongchai No. 1, Zhongchai No. 2, and Zhongchai No. 3) of *B. chinense* DC and one commercial variety (Zhonghongchai No. 1) of *B. scorzonerifolium* Willd. were released to meet the market requirement in China (Yao et al., 2013). All these varieties showed uniform performance in their agronomic traits and high saikosaponin content. However, due to the short domestication history of *Bupleurum* species, there are still challenging traits to deal with, specifically poor seed germination and viability loss in a short period.

The aim of this work was to develop a protocol to promote *Bupleurum* seed germination, thus making the cultivation of *Bupleurum* easier. The study involved the initial screening of hormones to identify a candidate that could promote the germination of Zhongchai No. 3 seeds, which is the newest released commercial variety. The effect of this optimized protocol on other varieties of *Bupleurum* species was also validated.

MATERIALS AND METHODS

Plant materials

Seeds of 'Zhongchai No. 3' were used in all studies, except in validation of the optimal protocol. The seeds used in the validation of the optimal protocol were those of the commercial varieties 'Zhongchai No. 1', 'Zhongchai No. 2' and the landrace 'Hubei chaihu' of *Bupleurum chinense* DC; the commercial variety 'Zhonghongchai No. 1' and the landrace 'Heilongjiang hongchaihu' of *B. scorzonerifolium* Willd; the landrace 'Rongxianzhuye' of *B. marginatum*; and the landrace 'B1' of *B. fruticosum*. All materials were kindly provided by Professor Jianhe Wei from the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences & Peking Union Medical College. All varieties were planted in the Sichuan province in 2011–2013, and the seeding rate was 0.6–0.75 million plants per hectare. The planting area of each variety was over 0.1 hectares. All seeds were harvested in November 2013.

Incubation condition and germination evaluation

One hundred seeds were placed on moist filter paper at the bottom of covered culture dishes (diameter, 10 cm). The filter in every culture dish was initially moistened with 6 mL of hormonal solution at different concentrations. Distilled water was added to a few dishes to avoid drying during seed incubation. All culture dishes were placed in an incubator. Temperature was maintained at 20°C. Emergence of seeds greater than 0.5 cm was considered as successful germination, at which point the germinated seed was removed from the culture dish. The cumulative germination percent was calculated for each dish. Germination index was calculated as:

$$GI = \sum (Gt/t)$$

Where, *Gt* indicates the germinated seed at the *t*th day.

Experiment 1: Candidate hormone screening

The hormones in this screening study were 6-benzyl aminopurine (6-BA), daminozide, gibberellin (GA3), paclobutrazol, uniconazole, and mepiquat chloride (MC). For each hormone, six concentrations (i.e., 0, 20, 40, 60, 80 and 100 mg $\cdot$ L^{-1}) were utilized. Each treatment consisted of two replicates.

Experiment 2: Critical GA3 concentration and duration of treatment

The promotion of germination of Zhongchai No. 3 seeds was insignificant in a preliminary experiment when the GA3 concentration was above 500 mg$\cdot L^{-1}$. Two sets of Zhongchai No. 3 seeds in 11 dishes were cultured in the presence of GA3 at concentrations of 0, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg$\cdot L^{-1}$. The results from the experimental trials varied significantly and, consequently, different quadratic and cubic regression models were fit to each experimental trial. Critical GA3 concentrations were calculated from the different models.

The optimal concentration of 230 mg$\cdot L^{-1}$ was used for the analysis of the duration of treatment for GA3. The seeds were incubated with GA3 for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 d. All seeds were incubated with distilled water following GA3 treatment for 10 d. The converse treatment sequence consisted of incubating the seeds with distilled water for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 d followed by GA3 treatment for 10 d. Then, all seeds were incubated with distilled water for 30 days.

Experiment 3: Validation of the optimal protocol

Seeds of 'Zhongchai No. 1', 'Zhongchai No. 2', 'Zhonghongchai No. 1', 'Hubei chaihu', 'Rongxianzhuye', 'Fengshun landrace', 'Heilongjiang hong chaihu' and 'B1' were incubated with 230 mg$\cdot L^{-1}$ GA3. Distilled water was utilized as the control. Three replicate experiments were conducted.

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 software (Norusis, 2008). Quadratic and cubic regression models were used to identify the critical incubation concentration of GA3. Duncan's multiple range test ($p < 0.05$) was used to compare the differences.

RESULTS AND DISCUSSION

To obtain the highest number of plants for the production of secondary metabolites, several studies based on micropropagation cultures of *B. fruticosum*, *B. chinense* DC, *B. scorzonerifolium* Willd, and *B. smishii* Wolff have been conducted (Fraternale et al., 2002; Li et al., 2008; Hao and Guan, 2012).

However, since the plants of *Bupleurum* species are small, the use of micropropagation cultures to industrialize the production of secondary metabolites is costly and has high labor requirements. Since plants of *Bupleurum* species can produce an abundance of seed, propagation from seed might be a more efficient method once the problem of poor germination is solved.

Seed germination represents the irreversible developmental-phase transitions from seed dormancy to germination in plants. These transitions involved

Table 1. Cumulative germination percentage and germination index of *Bupleurum* Zhongchai No. 3 seeds incubated after treatment with different hormones.

Hormones[a]	Cumulative germination percentage under different concentrations						Germination index under different concentrations					
	CK[b]	20 mg·L^{-1}	40 mg·L^{-1}	60 mg·L^{-1}	80 mg·L^{-1}	100 mg·L^{-1}	CK	20 mg·L^{-1}	40 mg·L^{-1}	60 mg·L^{-1}	80 mg·L^{-1}	100 mg·L^{-1}
6-BA	25.00	38.50	18.00	8.00	4.00	3.00	1.62	2.23	0.99	0.39	0.22	0.16
GA3	25.00	52.50	55.50	57.50	51.50	51.50	1.61	2.86	3.83	3.55	3.72	3.31
daminozide	25.00	16.00	17.00	22.00	13.00	11.00	1.60	0.95	1.05	1.28	0.87	0.67
MC	25.00	5.00	3.50	7.00	7.50	9.00	1.59	0.30	0.26	0.44	0.43	0.57
paclobutrazol	25.00	2.00	0.00	0.50	0.00	0.00	1.58	0.10	0.00	0.00	0.00	0.03
uniconazole	25.00	0.00	0.00	0.00	0.00	0.00	1.57	0.10	0.00	0.00	0.00	0.03

[a]Hormones are 6-benzyl aminopurine (6-BA), gibberellin (GA3), daminozide, mepiquat chloride (MC), paclobutrazol, and uniconazol
[b]CK is the concentration of 0 mg/L.

protein and hormonal alterations, especially the balance between abscisic acid and gibberellins (Graeber et al., 2010; Miransari and Smith, 2014).

A complex genetic network involved maturating genes, hormonal and epigenetic regulating genes may result this transitions (Bassel et al., 2011; Graeber et al., 2012). The germination of *Bupleurum* species seed is very difficult which considerably limits large-scale cultivation. This difficulty in germination might be attributed to seed maturity and germination inhibitors present in the seed coat (Wei et al., 2003). Dormancy of the harvested seeds also increases difficulty in germination (Hailu et al., 2008).

In the present study, the Zhongchai No. 3 seeds showed significantly different germination when treated with the different hormones (Table 1). Decreased germination was observed in the presence of daminozide, paclobutrazol, uniconazole, and MC. GA3 significantly promoted both cumulative germination percentage and germination index at all the concentrations used. In the case of 6-BA, the concentration of 20 mg·L^{-1} increased the cumulative germination percentage and germination index, which decreased when the concentration exceeded 40 mg·L^{-1}. Since the root growth of ‘Zhongchai No. 3’ seedlings was suppressed by 20 mg·L^{-1} 6-BA, we chose GA3 as the candidate hormone to further study germination promotion in *Bupleurum* species (Figure 1).

Gibberellins are diterpenoid, promoting germination and regulating plant growth. In previous studies, this plant hormone have been reported to stimulate the synthesis and accumulation of α-amylase, and regulate the expression of the *Osem* gene to control the production of one of the embryogenesis abundant proteins, resulting in the germination of seeds (Yamaguchi, 2008; Miransari and Smith, 2014).

Quadratic and cubic regression models were used to fit the GA3 concentration with cumulative germination percent and germination index (Table 2). Critical concentrations for promoting ‘Zhongchai No. 3’ seed germination were calculated from each model (Table 2, Figure 2). The predicted critical concentrations for increasing the germination index were in the range of 223.84–237.67 mg·L^{-1}, and the predicted critical concentrations for increasing the cumulative germination percentage were in the range of 230.21–238.78 mg·L^{-1}.

Therefore, the best GA3 concentration for promoting ‘Zhongchai No. 3’ seed germination might be 223.84–238.78 mg·L^{-1} When the ‘Zhongchai No. 3’ seeds were incubated with 230 mg·L^{-1}GA3, both the cumulative germination percentage and germination index were significantly higher than those of the control seeds when the incubation period was longer than 1 days (Table 3). However, a significant increase in cumulative germination percentage was observed when the converse treatment sequence was applied for more than 7 days, and a significantly increased germination index was observed when the duration of converse treatment was more than 9 days. These results indicated that incubation with an appropriate GA3 concentration on day 1 might be the most important factor in promoting ‘Zhongchai No. 3’ seed germination.

Significant germination variation was observed for other seed varieties. The commercial varieties showed a higher cumulative germination percentage and germination index than the landraces (Table 4). Only ‘Zhonghongchai No. 1’ and ‘Hubei chaihu’ seeds showed significantly increased cumulative germination percentages when the seeds were incubated with 230 mg·L^{-1} GA3. However, the increase in the germination index of ‘Hubei chaihu’ was insignificant. This finding indicated that different germination mechanisms exist

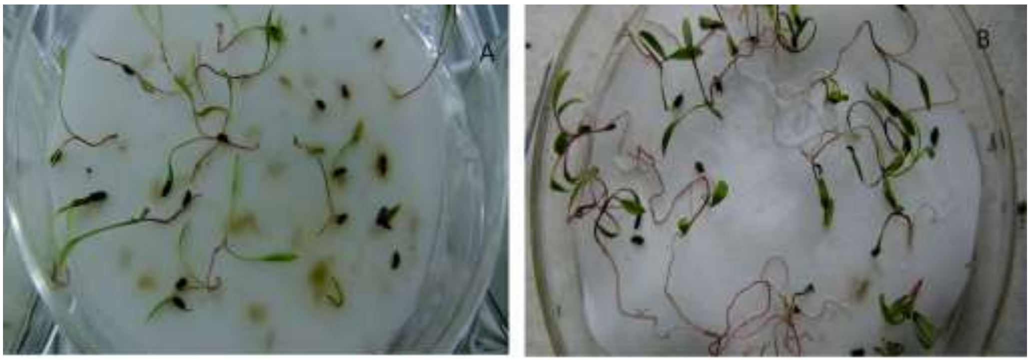

Figure 1. The effect of 6-benzyl aminopurine (20 mg · L^{-1}) and gibberellin (60 mg · L^{-1}) on seedlings of *Bupleurum* 'ZhongchaiNo. 3'.

Table 2. Quadratic and cubic models of cumulative germination percentage (GR) and germination index (GI) of *Bupleurum* for each experimental trial.

Trait	Regression equqations	R^2	C_0[a]
GI	$y=2.5906+0.03375x-7.1E-05x^2$	0.879	237.67
	$y=2.2826+0.04354x-0.00012x^2+6.8E-08x^3$	0.887	223.84
GR	$y=33.3811+0.4107x-0.00086x^2$	0.888	238.78
	$y=31.5524+0.4689x-0.00116x^2+4.1E-07x^3$	0.889	230.21

[a]C_0 is the Predicted optimum concentration.

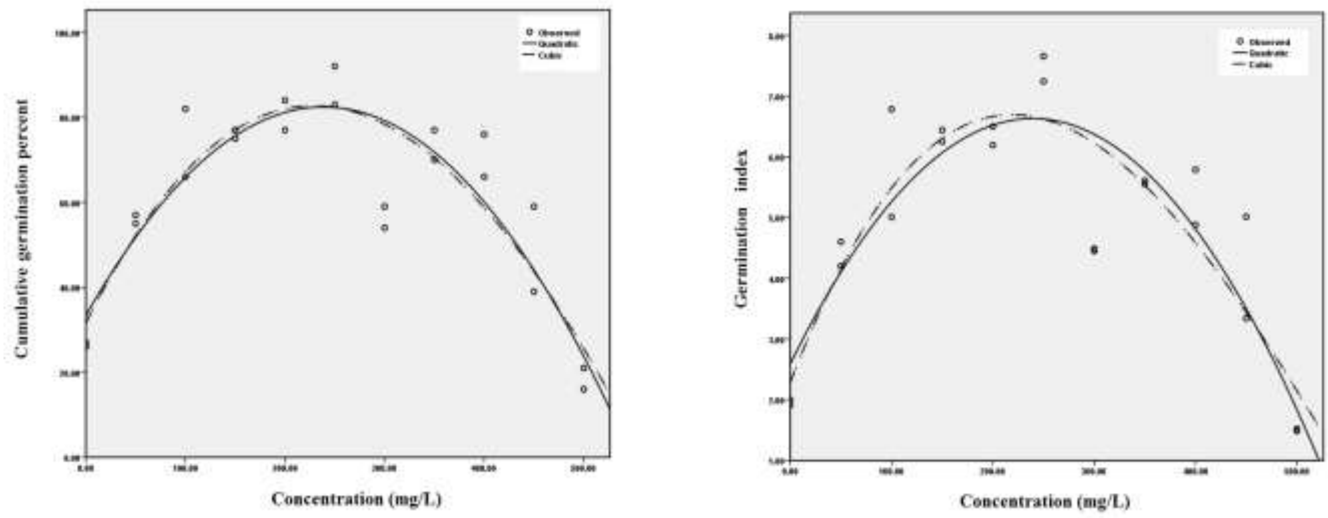

Figure 2. The quadratic and cubic correlation between gibberellin concentration with germination index and cumulative germination percentage of 'ZhongchaiNo. 3' .

among different varieties of *Bupleurum* species. This could explain why several reported protocols for promoting *Bupleurum* seed germination were ineffective in other studies (Yao et al., 2011).

Therefore, in the case of commercial varieties other than 'Zhonghongchai No. 1' and 'Hubei chaihu', protocols using different hormones or varying incubation temperatures should be explored. However, in our study, a change in temperature for germinating Zhongchai No. 2 seeds was ineffective (data not shown).

Since *Bupleurum* species seeds are sensitive to water and temperature and have a long germination period, direct seeding in the field is difficult. In addition, the plants of *Bupleurum* species grow slowly at the early stage, leading to considerable competition in the field between small plants and weeds. Such competition can be potentially avoided by centralized incubation in a greenhouse to increase seedling survival, followed by

Table 3. Cumulative germination percent (GR) and germination index (GI) of *Bupleurum* seeds under direct treatment and converse treatment by GA3.

Days	Direct treatment		Converse treatment	
	GR	GI	GR	GI
0	29.33[a]	2.01[a]	29.33[a]	2.01[a]
1	57.00[bc]	4.3[bc]	40.33[ab]	2.35[a]
2	55.00[bc]	4.06[bc]	47.00[ab]	2.87[a]
3	54.33[bc]	3.97[bc]	47.00[ab]	2.92[a]
4	59.00[bc]	4.35[bc]	49.33[ab]	3.01[a]
5	56.00[bc]	4.21[bc]	48.33[ab]	3.05[a]
6	56.67[bc]	4.19[bc]	42.67[ab]	2.61[a]
7	72.00[cd]	5.25[cd]	51.33[bcd]	3.30[abc]
8	57.33[bc]	4.14[bc]	55.33[bcd]	3.55[abc]
9	82.67[d]	6.13[d]	69.33[d]	4.98[c]
10	64.00[bcd]	4.73[bcd]	64[cd]	4.62[bc]

Table 4. The effect of gibberellin incubation on the germination percent (GR) and germination index (GI) *Bupleurum* varieties.

Varieties[a]	Spiecies	GA3 incubation (230 mg·L^{-1})		CK	
		GR	GI	GR	GI
ChuanhongchaiNo. 1	*B. scorzonerifolium* Willd.	81**	5.73*	52	5.05
Heilongjiang hongchaihu	*B. scorzonerifolium* Willd.	26	1.9	21.67	1.68
ZhongchaiNo. 1	*B. chinense* DC.	42	3.33	46	3.67
ZhongchaiNo. 2	*B. chinense* DC.	51	4.07	45	3.4
Hubeichaihu	*B. chinense* DC.	34**	1.17	10	0.32
Rongxianzhuye	*B. marginatum* Wallich ex de Candolle	24	1.47	18	1.32
B1	*B. fruticosum* L.	15	0.89	15	0.87

[a]'ZhongchaiNo. 1', 'ZhongchaiNo. 2', 'ZhongchaiNo. 3', and 'ZhonghongchaiNo. 1' are commercial varieties, the rest varieties are landraces in China. CK refer to Table 1.

transplanting of these seedlings into the field. Hence, the combination of GA3 treatment and centralized incubation might be a more efficient strategy for large-scale cultivation rather than direct seeding.

Conclusion

We found that treatment with 223.84–238.78 mg·L^{-1} GA3 during the first day best promotes *Bupleurum* 'Zhongchai No. 3' seed germination. This hormone treatment is only useful in the case of 'Zhonghongchai No. 1' and 'Hubei chaihu' seeds. The combination of GA3 treatment and centralized incubation could be an efficient way to industrialize the production of secondary metabolites from *Bupleurum* species. The future work should focus on the contents variation of secondary metabolites for *Bupleurum* plants under transplanting and direct seeding.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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