

The effect of controlled release fertilizer on growth, flowering, and leaf morphology of *Pulsatilla* species

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ABSTRACT

The effect of 14N-6.2P-11.6K controlled release fertilizer (CRF) on growth, flowering, and leaf morphology during vegetative growth of *Pulsatilla tongkangensis* and *P. cernua* var. *koreana* (*P. koreana*) were investigated. *Pulsatilla tongkangensis* flowered earlier (26 to 28 days) than *P. koreana* (50 to 51 days) after natural low-temperature treatment. The highest number of flowers was observed in *P. koreana* (3.6 flowers) and *P. tongkangensis* (1.7 flowers) with 0.9–1.2 g of CRF treatment. The formation of a third rachis and leaflet is a unique characteristic observed in *P. koreana*. Two morphological types of *P. tongkangensis* seedlings, with either long or short petioles, suggests that seedlings with long petioles are considered putative hybrids between *P. tongkangensis* and *P. koreana*. The development of axillary shoots in *P. koreana* seedlings before overwintering, when seedlings received more than 0.6 g of CRF, suggests that the seedlings undergo a phase change from juvenile vegetative growth to mature plants. This is the first detailed report describing morphology of leaves and plants during growth of seedlings and development of *Pulsatilla*.

Keywords: Controlled release fertilizer, juvenility, maturity, Pasque flower, *Pulsatilla cernua* var. *koreana*, *Pulsatilla tongkangensis*

INTRODUCTION

The genus *Pulsatilla* Mill. includes approximately 33 species native to Asia, North America, and Europe (USDA, Agricultural Research Service, National Plant Germplasm System, 2024). *Pulsatilla cernua* var. *koreana* (Y. Yabe ex) Nakai ex T. Mori (referred to as *Pulsatilla koreana*) (<http://www.ipni.org/ipni/idPlantNameSearch.do?id=711999-1>; accessed 22 August, 2023) is distributed across the Korean peninsula, growing in sunny areas with well-drained sandy soil. However, *Pulsatilla tongkangensis* Y. N. Lee et T. C. Lee (<https://www.ipni.org/n/20005221-1>; accessed 22 August, 2023) is growing limited regions in Korea, specifically near calcified rock cavity cliffs and in well-drained areas (Lee *et al.*, 2010; Oh *et al.*, 2014; Yoo *et al.*, 2009).

The application of CRFs such as 14N-6.2P-11.6K Osmocote (<https://iclgrowingsolutions.com/en-us/ornamental-horticulture/products/osmocote-14-14-14-3-4-m/>; accessed on 23 September, 2023) offers a time-efficient alternative to conventional liquid fertilizers (Hauck, 1985). However, previous studies with *P. vulgaris* Mill. grows in

nitrogen-poor soils (Kellner, 1993) have not examined the effect of CRF treatment during the vegetative growth of seedlings or changes in leaf morphology and flowering in the two *Pulsatilla* species studied in this report.

Growth and flowering responses, leaf morphology during seedling stage and leaf analysis for macro- and micro-elements in two species studied in this report has not been reported to our best knowledge. Flowering was accelerated, and the number of flowers was increased in 'Rainier White F1' (*Cyclamen persicum* Mill) Persian cyclamen promoted earlier flowering and increased the number of flowers and buds with treatment of Osmocote 6M (Bosiacki *et al.*, 2021).

Leaf and flower morphology are important characteristics for identifying *Pulsatilla* species (Lee *et al.*, 2010). The formation of the third rachis in *P. koreana* serves as a distinguishing morphological characteristic, to provide a clear distinction between these two species (Lee *et al.*, 2010). During the vegetative phase after germination, morphological changes occur in leaf size and shape, rachis length, and trichome distribution. Identifying vegetative or adult leaves was reported in *Arabidopsis* (Manuela and Xu, 2020). In *Verbascum blattaria* Mill., trichomes present only on seed pods and bracts but absent in leaves at the seedling stage may not be useful for identification or characterization of unknown species (Kim *et al.*, 2023).

The objectives of this research are to (a) examine the morphology of leaves and plants of *P. koreana* and *P. tongkangensis* and variations among seed-propagated *P. tongkangensis* populations and (b) study the effect of CRF treatments on the growth and flowering response.

MATERIALS AND METHODS

Seed germination and transplanting of seedlings: Open-pollinated seeds of *P. koreana* acquired from Mokpo National University in Muan County, Chonnam Province and of *P. tongkangensis* seeds collected from Jungsun, Kangwon-do Province, Korea used in this experiment. On 1 July, 2023, *P. koreana* seedlings developed 3 to 4 true leaves and were transplanted into 1800 square band pots (9.0 × 9.0 × 11.5 cm, Anderson Die & Manufacturing, Portland, OR, USA). On 6 July, 2023, *P. tongkangensis* seedlings with similar leaf development were transplanted into JānorPot 3D containers (9.0 × 9.0 × 10.0 cm, Summit Plastic Co., Mogadore, OH, USA).

Controlled release fertilizer treatments: On 6 July, 2023, CRF was top-dressed at rates of 0, 0.3, 0.6, and 1.2 g per pot for *P. tongkangensis* and 0, 0.3, 0.6, 0.9, and 1.2 g per pot for *P. koreana*. The CRF granules were covered with an additional growing medium to ensure they were not visible. On 6 August, 2023, the pots were spaced at a 9 × 9 cm distance to prevent the leaves adjacent plants from touching. On 25 September, 2023, CRF was reapplied at the same rates.

Observation of leaf and plant morphology: On 17 September, 2023, the leaf morphology of both *Pulsatilla* species was observed when the seedlings had formed 5 to 6 true leaves. The number of leaflets per leaf and the shape of the leaflets were recorded according to previously described (frame A, Fig. 1; Lee *et al.*, 2010). Briefly, the third leaflets and rachis as indicated with solid circle indicated in leaves 2, 3, and 4 in *P. koreana* were observed. However, these was not observed in *P. tongkangensis*. Only the second rachis indicated with a solid arrow in *P. tongkangensis* were formed, while the third rachis indicated with a dotted arrow in *P. koreana* was formed (Fig. 2).

Based on the formation of the third leaflet and rachis, the morphologies of *P. tongkangensis* and *P. koreana*, as well as an intermediate form between these two species, especially in

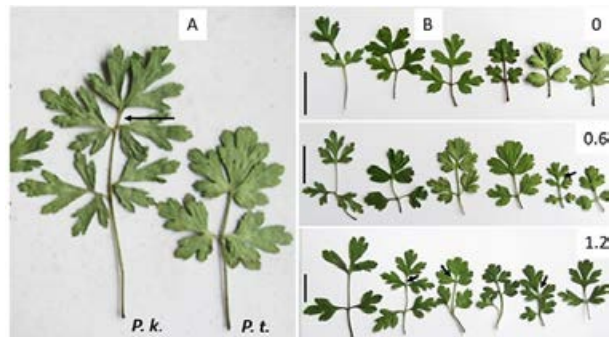


Figure 1. Typical leaf morphology of *P. cernua* var. *koreana* (*P. k.*) and *P. tongkangensis* (*P. t.*); Frame A: formation of (arrow) in *P. koreana* and absence of the third leaflet and rachis in *P. tongkangensis*. Frame B: *Pulsatilla tongkangensis* plants that formed the third rachis (solid arrow) when seedlings were treated with 0, 0.6, and 1.2 g 14N-6.2P-11.6K Osmocote. These leaves were collected for analysis of macro- and micro-elements before overwintering. Bar = 2 cm

P. tongkangensis that are produced, especially when plants received 0.6 and 1.2 g CRF were recorded (frame B, Fig. 1). On 26 October, 2023 the number of plants classified based on leaf morphology was recorded (Table 2). Short and compact leaves developed at the crown of the seedlings of *P. koreana* (C2) and of *P. tongkangensis* (T2) that received CRF treatments were counted (Fig. 4).

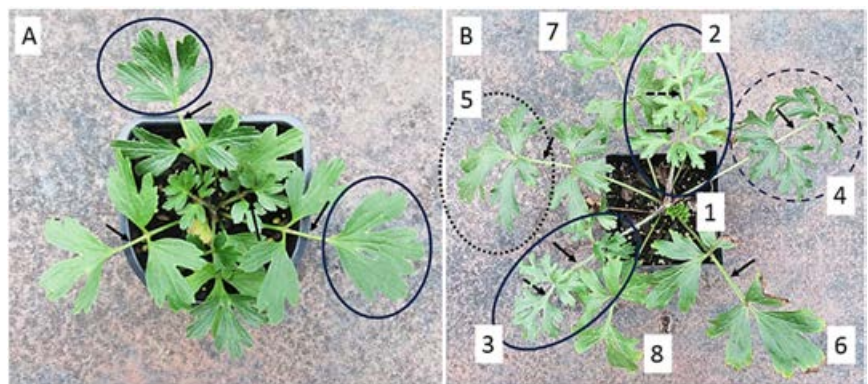


Figure 2. Morphological differences of *P. tongkangensis* and *P. koreana* on 17 September, 2023. (A) *P. tongkangensis* leaves exhibit only two leaflets with the second rachis (solid arrow) and leaflet (solid circle), (B) *P. koreana* leaves show a well-developed third leaflet attached to the third rachis (dotted arrow) at the proximal end of the second rachis (solid arrow) in leaves 2 and 3 (indicated in the square box). The third rachis is not fully developed in leaf 4 and is absent in leaves 5 through 8, counted from the youngest leaf (leaf 1)

Experiment design and statistical analysis: Following the overwintering, all pots were completely randomized and designed on a wooden deck 45 cm above the ground until the completion of the experiment. The dates of flowering for the first and second flowers, originating from both the main and axillary shoots, were recorded, and the total number of flowers, was recorded two weeks after the anthesis of the first flower bud from the main shoot. The number of days to flower was counted from 6 February. The monthly average temperatures from sowing in July 2023 to March 2024 were tabulated using data from Weather Underground (<https://www.wunderground.com/forecast/KARB>) (Table 1).



Figure 3. Influence of 14N-6.2P-11.6K CRF on growth morphology of *P. tongkangensis* on 17 September, 2023. (A) CRF was applied at rates of 0 g (1), 0.3 g (2), 0.6 g (3), 0.9 g (4), and 1.2 g (5) per pot on 6 July, 2023. (5-A) Short leaf blade and (5-B) long leaf petiole. Two distinct types of *P. tongkangensis* that received the same amount of CRF. Leaf length was measured from the center of the plant (crown) to the tip of the leaf. (B) The image depicts the shoot apex, including the main (Ma) and axillary (Ax) shoot.

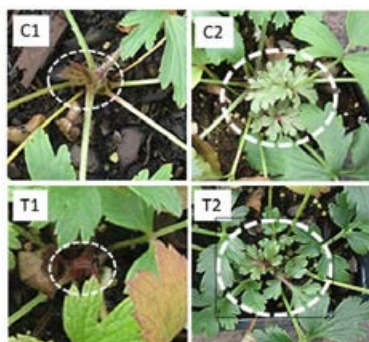


Figure 4. Overview morphology of seedlings prior to overwintering for *P. cernua* var. *koreana* (C1 and C2) and for *P. tongkangensis* (T1 and T2) that formed short and compact leaves. Seedlings received 0g (C1 and T1) or 0.9g 14N-6.2P-11.6K Osmocote (C2 and T2). Photographed on 1 October, 2023

As the temperatures decreased around 15 October, 2023, reaching 1.7 °C at midnight on 31 October (Table 1), the seedlings in the pots were prepared for overwintering. Pots were buried in a ground bed and watered. The seedlings were grown until 20 November, 2023 after which all pots were covered with approximately 5 cm of grass clippings and chopped dried leaves processed with a lawn mower.

Table 1. Monthly high. average, and low temperature (°C) from July, 2023 to March, 2024, and indoor temperature from March to April, 2024

Year	Month	Max.	Ave.	Low.	Indoor
2023	July	26.6	19.1	13.8	
	August	25.9	18.89	13.0	
	September	21.5	14.5	10.1	
	October	13.2	8.3	3.8	
	November	7.9	2.5	-2.8	
	December	5.6	2.2	-1.4	
2024	January	0.5	-2.0	-6.0	
	February	6.8	1.4	-3.9	5.5
	March	10.6	4.6	-0.8	6.1

All pots were lifted from the ground on 6 February, 2024. To protect the seedlings from frost, they were moved to a non-heated garage from 19:00 to 08:30 when temperatures dropped below $-2\text{ }^{\circ}\text{C}$ during the night. The temperatures were recorded at 08:30. The experiment, including the recording of flower bud development and flower counts, and morphological characteristics was completed on 1 April, 2024 (Table 2). Statistical analyses were performed using the Excel software program (Microsoft Corporation, 2019).

RESULTS AND DISCUSSIONS

Growth and flowering responses: *Pulsatilla tongkangensis* flowered significantly earlier (26 to 28 days) compared to *P. koreana* (50 to 51 days) (Table 2). The days to flowering were unaffected by CRF treatments in both species. Osmocote 6M treatment accelerated the flowering of Cyclamen (Bosiacki *et al.*, 2021). No flowers were produced from the axillary shoots treated with 0 and 0.3 g CRF in both species. Application of 0.6 to 0.9 g of CRF increased the number of total flowers, ranging from 0.8 to 3.6 flowers in *P. koreana* and from 0.6 to 1.7 flowers in *P. tongkangensis*. This increase was attributed to the formation of axillary shoots that produced flowers, producing 1.6 flower buds from the axillary shoot. The flowering percentage exceeded 80% in both species. When seeds were sown in July, 7-month-old plants treated with 1.2 g CRF produced up to four flowers per plant, two flowers each from the main and axillary shoots as observed after overwintering. Osmocote 6M treatment increased the number of flower buds of Cyclamen (Bosiacki *et al.*, 2021). Therefore, enhancing the potential for axillary shoot formation before overwintering (Fig. 3) and continued development over the winter (Fig. 5) could be beneficial for increasing the number of flowers.

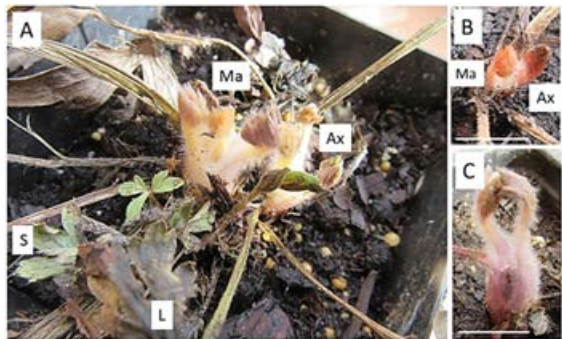


Figure 5. Overall appearance of *Pulsatilla* plant treated with 1.2 g 14N-6.2P-11.6K CRF after overwintering. (A) Main shoot (Ma) and axillary shoot (Ax) with long leaves that decayed (L) and short and compact leaves that remain green (S). (B) Appearance of new shoots of *P. koreana* that were not treated with CRF, showing only the Ma. (C) New shoot of *P. tongkangensis* that were not treated with CRF. Photographed on 7 February, 2024. Scale bar = 1 cm

Morphology of leaves and shoot apex: *Pulsatilla tongkangensis* leaves were ternate with palmately lobed leaflets and a cordate base (Fig. 1; indicated by the circle in frame A, Fig. 2). In contrast, *P. koreana* had oddly pinnately compound leaves (Fig. 1; indicated by the dotted circle, leaf no. 5 in frame B, Fig. 2). The formation of the third rachis and third leaflet was observed only in *P. koreana* (dotted arrow, leaf Nos. 2, 3, and 4 in frame B, Fig. 2). The third rachis and leaflet were absent in older leaves numbered 5, 6, 7, and 8, counted from the crown of the *P. koreana* seedlings. Most *P. tongkangensis* seedlings did not form the third rachis and leaflet when they were not treated with CRF (frame B, Fig. 2). This morphological change could signify a phasic shift from vegetative growth to reproductive development influenced by CRF application. Therefore, the presence of a

third leaflet may not serve as a definitive morphological characteristic for identifying and characterizing *P. tongkangensis* potential hybrids with *P. koreana*, and accurate identification should ideally involve molecular markers (Lee *et al.*, 2010).

Pulsatilla. tongkangensis seedlings exhibited significantly shorter petioles (frame A, Fig. 1; Table 2) and wider leaflets (data not recorded) compared to *P. koreana* (frames A and B, Fig. 2). For *P. koreana* seedlings treated with 0.6 g of CRF, the longest leaf measured 7.38 cm, which was significantly longer compared to the control (4.98 cm). However, leaves treated with 1.2 g of CRF were shorter (6.5 cm) compared to those treated with 0.6 g (Table 2). In *P. tongkangensis*, the longest leaf length of 3.77 cm in seedlings treated with 1.2 g of CRF was significantly greater than that of the control (2.43 cm) and those receiving 0.3 g of CRF (2.73 cm).

However, two types of petiole length patterns were observed among *P. tongkangensis* seedlings treated with 1.2 g of CRF: leaves with either a short petiole (5-A in frame A, Fig. 3) or a long petiole (5-B in frame A, Fig. 3) may suggest that *P. tongkangensis* plants with long petiole (intermediate morphology) could result from the hybridization with *P. koreana* and this pattern was only observed in *P. tongkangensis* as two to three plants showed intermediate morphology (Table 2).

In both species, axillary shoot (Ax) before overwintering was not observed when seedlings were not treated with CRF. However, *P. tongkangensis* seedlings that received 0.6 and 0.9 g CRF, and in *P. koreana* was also observed when seedlings were treated with 0.3 to 0.6 g CRF developed an axillary shoot adjacent to the main shoot (Ma) on 17 September, 2023 (frame B, Fig. 3). The formation of axillary shoots, influenced by the application CRF treatments suggests that the shoot apex of the main shoot may undergo reproductive development and similar findings have been reported for *P. vernalis*, where a single application of ammonium fertiliser resulted in increased shoot production and enhanced flower bud formation (Kellner, 1993).

On 1 February, 2024, after overwintering, newly developing leaves of *P. tongkangensis* plants that had received 0.9 g CRF emerged above the ground. The short and compact leaves (S) indicated by a dotted circle (frames C2, T2, Fig. 4) remained semi-green when the pots were lifted (Frame A, Fig. 2). Upon lifting the plants from the ground, the shoot apices of *P. koreana* that had not been treated with CRF (frame B, Fig. 5) appeared smaller and less developed compared to those of *P. tongkangensis*, which showed two well-developed leaves (frame C, Fig. 5). In *P. koreana*, two shoot apices of similar development was observed. However, plants treated with 1.2 g of CRF exhibited more advanced development of both the main and the axillary shoots.

The presence of short and compact leaves developed before overwintering and the formation of axillary shoots may suggest that the seedlings are transitioning towards maturity before overwintering. Therefore, the term 'seedling' is applied before overwintering, while 'plant' is used following overwintering. Flower bud development in young *Pulsatilla* plants may occur later than mid-July and mid-August observed in older plants (Wang and Bartholomew, 2001). Reproductive competence in *Pulsatilla* species may progress in response to short-day photoperiods and low-temperature environments before overwintering. Once flowering occurs in early spring, no further flowering was observed in both *Pulsatilla* species. This suggests that short photoperiods and lower temperatures facilitate the transition from vegetative growth to reproductive development, keeping plants vegetative under long days and high temperatures as reported in June-bearing strawberries (*Fragaria ×ananassa*, Duch.) (Black *et al.*, 2008). Therefore, under natural environmental conditions, floral bud initiation in *Pulsatilla* likely occurs in early autumn when photoperiod (12 – 13.5 hours) and temperature (18/16°C) become conducive for floral induction (Black *et al.*, 2008). Further experiments are required to understand the critical photoperiod and temperature for floral induction and development.

Table 2. Effect of 14N-6.2P-11.6K Osmocote (CRF) on the growth and development of *Pulsatilla koreana* and *Pulsatilla tongkangensis*

<i>Pulsatilla</i> species	14N- 6.2P- 11.6K Osmo- cote (g)	Data collected before overwintering in 2023				Data collected at flowering after overwintering in 2024		
		Long- est leaf length (cm)	No. of plants with Ax ^a	Formati on of mature leaves ^b	Plant type based on leaf morphology ^c	No. of flowers		Flower- ing %
						First / second inflores- cence ^d	Total	
<i>P. koreana</i>	0	4.98	0	5 N	5 K	0.8 / 0	0.8	80
	0.3	6.14	1	3 Y/2 N	5 K	2.0 / 1.4	3.4	100
	0.6	7.38	2	5 Y	5 K	2.0 / 1.6	3.6	100
	1.2	6.50	2	4 Y / 1 N	5 K	2.0 / 0.8	2.8	80
	HSD 1% ^e	1.735	- ^f	-	-	-	1.13	-
<i>P. tong- kangensis</i>	0	2.43	0	na	4 T / 2 I	0.6 / 0	0.6	67
	0.3	2.73	0	na	3 T / 3 I	0.8 / 0	0.6	67
	0.6	3.45	1	na	3 T / 3 I	1.0 / 0.3	1.3	100
	0.9	3.58	2	na	4 T / 2 I	1.3 / 0.2	1.5	83
	1.2	3.77	2	na	3 T / 3 I	1.5 / 0.2	1.7	83
	HSD 1% ^e	0.893	-	-	-	-	0.85	-

^a Refer to Figs. 3 and 5 for the main shoot (Ma) and axillary shoot (Ax).
^b Number of seedlings with mature leaves indicated with an arrow (black solid) and leaves 2, 3, and 4 in solid and dotted circles (Fig. 2). Indicated as yes (Y) or no (N). Mature leaves were not developed (na).
^c Number of *P. koreana* type (K), *P. tongkangensis* type (T), and intermediate type (I) seedlings exhibiting mature/immature leaf morphology characteristics. For example, 4 Y/1 N indicates that four plants formed mature leaves and one plant did not, and “4 K/1 I” indicates that four plants showed *P. koreana* type and one plant intermediate type. Refer to Fig. 1.
^d Number of first / second flowers developed both from the primary shoot and from the axillary shoot apex.
^e Tukey’s honestly significant difference (HSD) test at $p \leq 0.01$.
^f Not analyzed.

CONCLUSIONS

The flowering of *P. tongkangensis* occurred earlier (26 to 28 days) than that of *P. koreana* (50 to 51 days); however, the flowering time was not significantly affected by CRF treatment in either species. The formation of the third rachis and leaflet, which is a distinct morphological characteristic of *P. koreana*, implies a transition from vegetative growth to reproductive development, which includes the formation of axillary shoots and compact leaves in response to low temperatures and short-day photoperiods before overwintering. The long petioles observed in *P. tongkangensis* seedlings suggest that these seedlings were hybrids. We first reported on the leaf morphology before and after overwintering and axillary shoot formation in relation to the number of flowers. Formation of third rachis and leaflet in *P. tongkangensis* as influenced by CRF treatment required molecular work to identify whether these plants with long petioles are interspecific hybrids.

Acknowledgements

Germplasm used in this project was provided by J.W. Kang, D.W. Suh, Y.K. Yoo, and M.C. Chung. We express our sincerely thanks for their contributions.

This research was supported by the research grant awarded to Y.H. Joung from the New Breeding Technologies Development Program (Project No. RS-2024-00322366) through the Rural Development Administration (RDA), Korea.

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