**Overcoming root dormancy and identifying the storage
behaviour of *Lilium polyphyllum* seeds**

Journal:	<i>Botany</i>
Manuscript ID	cjb-2018-0161.R3
Manuscript Type:	Note
Date Submitted by the Author:	07-Nov-2018
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Keyword:	conservation, critically endangered, seed dormancy, seed storage, seedling production
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

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Manuscripts

(i) Full title of the paper

Overcoming root dormancy and identifying the storage behaviour of *Lilium polyphyllum* seeds

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25 **Title of paper:**

26 **Overcoming root dormancy and identifying the storage behaviour of *Lilium polyphyllum***
27 **seeds**

28 **Abstract**

29 *Lilium polyphyllum* is a critically endangered medicinal herb of Himalaya. Seeds have epicotyl
30 morphophysiological dormancy, and in the field a minimum of 34 weeks is required for root
31 emergence. To date, treatments to decrease the time for root protrusion and seed storage
32 behaviour have not been determined. We evaluated the effects of plant growth regulators and
33 nitrogenous compounds and seed scarification on root emergence and determined the storage
34 behaviour of seeds. Root emergence at 20°C was significantly increased by 100 ppm IBA,
35 KNO₃ and thiourea and by application of NaOCl solution for 30 min. Mean germination time
36 was decreased significantly by 50 ppm GA₃ (28 days) compared to 100 ppm GA₃ (36 days).
37 After 12 months of dry storage, viability of seeds (TTC method) at room temperature (10-35°C),
38 0-4°C and -20±1°C was 95%, 100% and 100%, respectively; thus, seeds have orthodox storage
39 behaviour. Our recommendation for seedling production is to store seeds at room temperature
40 until they can be sown under warm wet conditions. Roots emerge after about 8 weeks, and then
41 seeds should be given a 2-week cold moist treatment to break shoot dormancy. After 2 weeks of
42 cold, transfer seeds to warm (20°C) spring temperatures to promote growth.

43 **Keywords:** conservation; critically endangered; seed dormancy; seed storage; seedling
44 production

45

46 **Introduction**

47 Seed germination and seedling establishment are critical stages in the life cycle of plants
48 growing in their natural habitats (Fenner and Thompson 2005; Baskin and Baskin 2014), and
49 have great importance for species fitness. Hence, understanding the dormancy, germination and
50 seedling establishment are necessary for developing long term conservation strategies especially
51 for threatened species. Information on seed germination and seedling establishment
52 requirements is needed to artificially propagate the seedlings of endangered species for
53 successful augmentation, introduction and reintroduction programmes (Shen et al. 2010). Target
54 eight of Global Strategy for Plant Conservation (GSPC) aims to include at least 75% of the
55 threatened plant species in ex situ collections, preferably in the country of origin by 2020. Ex
56 situ seed conservation is an effective way to safeguard threatened plants from extinction
57 (Teixido et al. 2017), and it is a practical way to preserve large amounts of genetic material in a
58 small space with minimum risk of damage. Further, information on seed storage behaviour is
59 needed to determine whether seeds stored in genebanks remain viable, because seed viability
60 and germination are key factors in the success of restoration efforts, especially when stored
61 seeds are used (Bhatt et al. 2018).

62 *Lilium polyphyllum* D. Don ex Royle, a critically endangered Himalayan species, occurs
63 at an elevation of 2200-3200m a.s.l. in Western Himalaya (30°.42'N, 78°.25'E to 30°.99'N,
64 78°.94'E), and it inhabits the understory of coniferous forests dominated by *Cedrus deodara*.
65 Leaf emergence from the bulb begins in March in Garhwal Himalaya, India. Approximately two
66 white speckled pink flowers per plant are produced, and rarely several (32 in garden, A. Dhyani,
67 pers. obs.), and the flowering period is 15-20 days during June-July. Numerous seeds (average

100 per capsule) are produced, during September-October. Seedlings produce increased leaf numbers by repeating the growth pattern annually, and bloom after 7-8 years in Himalayan temperate coniferous forest. A mature mother bulb develops small bulblets during the vegetative growth period (prior to flowering), and it decays after several seasons of flowering leaving a few daughter bulbs (Dhyani, unpublished).

Lilium polyphyllum bulbs contain phenolic compounds, i.e. phloridzin, caffeic acid, chlorogenic acid, 3-hydroxy cinnamic, rutin, gallic acid, ferulic acid, vanillic acid, 3-hydroxy benzoic (Giri et al. 2017). The bulbs have been used in more than 30 Ayurvedic medicinal formulations, as an aphrodisiac, astringent, antipyretic, expectorant, galactagogue, revitalizing tonic and to treat general debility (Warrier et al. 1997). Indiscriminate harvesting of bulbs, small population size, narrow geographical range, small area of occupancy, habitat degradation and continued decline in mature individuals of *L. polyphyllum* are responsible for the inclusion of the species under Critically Endangered status in IUCN Red List (Dhyani et al. 2018).

Seeds of *L. polyphyllum* have an underdeveloped (small) embryo and it must grow inside the seed prior to germination (morphological dormancy MD). The embryo also has physiological dormancy (PD). That is, the seeds have morphophysiological dormancy (MPD) (Dhyani et al. 2013). Seeds are dispersed in autumn (October, <15°C), and it takes about 34 weeks for the cotyledonary petiole/sheath (hereafter root) to emerge. The primary root emerges from the root-shoot axis (which is carried outside the seed *via* the cotyledonary petiole/sheath) after the cotyledonary petiole/sheath has emerged from the seed. Roots emerge during June (19°C), and a bulblet is produced in July. The bulblet requires a cold (0-4°C) moist treatment for dormancy break, and a leaf (shoot) emerges from it in mid-March to April (14°C) of the

following year, i.e. the second spring after seed dispersal. Thus *L. polyphyllum* seeds take approximately 18 months from seed dispersal to complete germination (leaf emergence) in Himalayan temperate coniferous forest of Garhwal, India (Dhyani et al. 2010). That is, seeds have epicotyl MPD (Dhyani et al. 2013).

Presently, nine different levels of morphophysiological dormancy (MPD) have been identified, based on temperature requirements for embryo growth, the breaking physiological dormancy and emergence of the root and shoot, and on the response of seeds to GA₃ (Baskin and Baskin 1998; Baskin et al. 2008). One of the levels is deep simple epicotyl MPD in which considerable embryo growth occurs prior to root emergence. In deep simple epicotyl MPD warm treatment (moist at $\geq 15^{\circ}\text{C}$) is required to break the PD of the root and cold treatment (moist at ca. 0 to 10°C) is required to break PD of the epicotyl. Similar to *L. polyphyllum*, in seeds of *Asarum canadense* (Baskin and Baskin 1986), *Erythronium japonicum* (Kondo et al. 2002), *Gagea lutea* (Kondo et al. 2004), *Narcissus hispanicus* (Copete et al. 2011), PD of the root is broken by warm moist treatment, and PD of the epicotyl is broken by cold moist treatment.

One objective of this study on *L. polyphyllum* was to find a way to decrease the time required for root emergence, i.e. to either break PD or remove the mechanical resistance of the embryo covering tissues on growth of the embryo. Since treatment with plant growth regulators and nitrogenous compounds and scarification are known to overcome PD and promote germination (Bewley and Black 1994; Baskin and Baskin 2014), we hypothesized that these treatments would overcome PD of the root and thus decrease the time required for root emergence in seeds of *L. polyphyllum* known to have epicotyl MPD.

A second objective was to determine the seed storage behaviour of this rare species. That is, knowing how to maintain the viability of seeds, would facilitate storing them in a genebank. The storage behaviour of seeds can be categorised as orthodox, recalcitrant and intermediate (Roberts 1973; Ellis et al. 1990, 1991). Orthodox seeds have low moisture content (MC) after dispersal and can be dried to 5%, or lower, and stored at subzero temperatures (optimum= -18°C) for long periods without loss of viability. However, freshly matured recalcitrant seeds have a high MC and lose viability rapidly if they are dried to a MC of less than 20-30%. Further, they cannot be stored at temperatures below 0°C. Seeds with intermediate storage behaviour survive a MC of ~6-12% and can be stored at cool (10-0°C) temperature; however, subzero temperatures rapidly reduce viability in these seeds. Seeds of *L. polyphyllum* live in the field in the Himalayas from dispersal time in September-October to the following June when it is warm enough for PD of the root to be broken, after which it emerges. Since seeds in the field survive exposure to low winter temperatures before root dormancy is broken, we hypothesized that they have orthodox storage behaviour.

Materials and Methods

Seed harvesting and handling

Ripe seeds of *L. polyphyllum* were harvested during the first week of October 2008 in Dhanaulti (2200m a.s.l., 30°.42'N, 078°.25'E) in (Western Himalaya) the Uttarakhand state of India. The plants were growing under the dense canopy of coniferous woodlands. We sampled 25 plants for seed collection, and each plant had approximately two capsules with 100 seeds/capsule. Mature capsules were dried for one week at ambient room temperature, after

which seeds were removed manually from them and used in experiments after 5 days of drying at room temperature. The criterion for seed germination in this study was emergence of the cotyledonary petiole/sheath, which we will refer to as “germination” or as “root emergence”.

Effect of plant growth regulators, nitrogenous compounds and seed scarification on root emergence

Seeds were soaked in concentrations of 50 and 100 ppm of the plant growth regulators gibberellic acid (GA₃) and indole butyric acid (IBA), and in nitrogenous compounds potassium nitrate (KNO₃) and thiourea (CH₄N₂S) for 24 hrs. Previous studies revealed that seeds were water-permeable (Dhyani 2012). Seeds were scarified by soaking them in sodium hypochlorite (NaOCl, 4% available chlorine) for 30 and 45 minutes; seeds soaked in distilled water for 24 hrs served as the control. For each treatment, three replicates of 48 seeds were used. After all treatments, seeds were sown at a depth of approximately 1cm in styrofoam trays containing a mixture of soil, sand, and litter in 1:1:1 proportion and placed in a non-temperature-controlled greenhouse (hereafter referred to as the ‘greenhouse’) at Srinagar Garhwal (550 m a.s.l.). The day and night temperatures in the greenhouse were about 28 and 12 °C, respectively, (average of 20°C); 20°C had previously been shown to be a favourable temperature for root emergence (Dhyani et al. 2013). The soil was moistened, as required, with running tap water to keep the surface moist. Germination was monitored weekly for two growing seasons. Seeds were lifted manually and examined for root emergence, and germinated seeds were moved to other trays. The germination percentage was calculated by dividing the number of germinated seeds by the total number of sown seeds in each tray and mean germination time (MGT) was calculated as

per Ellis and Roberts (1981). MGT is a measure of the rate of germination and of the sharpness of the germination peak.

Effect of storage conditions on seed viability

The moisture content (MC) of seeds was determined by the oven-drying method, *i.e.* 103°C for 17 hrs (ISTA 2012). Fresh seed viability was tested using 2,3,5- triphenyl-2H-tetrazolium chloride (TTC) prior to commencement of experiments (ISTA 2012). Three replicates of 20 seeds were incubated on moist filter paper at room temperature for 24hrs and then cut along the longitudinal axis with a razor blade. Both seed sections were incubated in a 0.1% aqueous solution of TTC for 24 hrs at 37°C in darkness. Strongly red-stained seeds were considered to be viable.

To determine the effect of storage conditions on seed viability, seeds were placed in airtight polythene bags to maintain the original MC and stored at different temperatures: (i) deep freezer (-20±1°C), (ii) refrigerator (0-4°C), and (iii) room temperature (10-35°C). At 3-month intervals for 1 year, viability of stored seeds was tested by the TTC method and seed MC was determined, as described above.

One way ANOVA with LSD (Least significant difference) at 5% level of significance were used to analyse the effect of different plant growth regulators, nitrogenous compounds and scarification on germination and mean germination time. The Tukey-Kramer test was used to compare means of germination percentage and mean germination time between treatments when significant differences were found. All analyses were conducted in SPSS 19.0 software.

Results

Effect of plant growth regulators, nitrogenous compounds and seed scarification on root emergence

Plant growth regulators, nitrogenous compounds and scarification with NaOCl had significant effects on germination ($P < 0.05$, Figure 1). Germination of seeds treated with GA₃ and 50 ppm IBA did not differ significantly from that of the water control. The highest germination (50.6%) was obtained from seeds treated with NaOCl for 30 min, but germination after this treatment did not differ significantly from that of seeds treated with 100 ppm IBA, KNO₃ and, thiourea or 45 min scarification with NaOCl. However, treatment of seeds with 100 ppm IBA, KNO₃, thiourea or NaOCl significantly increased germination compared to the water control and seeds treated with GA₃. Approximately 50% of the seeds treated with NaOCl for 30 min did not germinate. Further, 58-75% of the seeds treated with plant growth and nitrogenous compounds and 75% of the control seeds failed to germinate (Figure 2). However, 50 ppm GA₃ significantly reduced MGT (28.7 days) compared to 100ppm GA₃ (36.1 days).

Effect of storage conditions on viability

At time of collection, 100% of the seeds were viable. Viability of seeds stored at room temperature decreased by 97% after 9 months, but it remained unchanged for seeds stored in the refrigerator and deep freeze. After 12 months storage at room temperature (10-35°C), 0-4°C and -20±1°C, seed viability was 95, 100 and 100%, respectively, and MC was 6.8, 8.8 and 9.8 %, respectively (Table 1).

Discussion

Freshly matured seeds of *L. polyphyllum* are dormant. Before the root can emerge, the small embryo grows, and PD of the root is broken. Since both the root and the shoot have PD, the embryo has low growth potential and cannot overcome the mechanical resistance of the endosperm and seed coat. Thus, scarifying seeds of *L. polyphyllum* with NaOCl (Figure 1) and either removal of the seed coat or mechanically scarifying the seed coat on the outer three margins without disturbing embryo enhanced germination 50.6, 50 and 91%, respectively (Dhyani et al. 2014). Scarification or removal of the seed coat reduces the mechanical resistance to germination and thus allows the embryo to grow and the root to emerge. Germination of dormant seeds of *Lilium martagon* var. *cattaniae* also was enhanced by removing the seed coat (Paric et al. 2008). Further, embryo excision accelerated seed germination in 28 *Lilium* species and varieties (Pelkonen 2005). In *L. polyphyllum* seeds under natural conditions, however, PD of the root is broken during warm moist conditions in summer, after which the root emerges.

The beneficial effects of IBA and KNO₃ on germination were not investigated, but we assume they played some role in increasing the growth potential of the embryo. In addition, IBA is known to promote the formation of roots (Hartmann et al. 2002). In an earlier study on *in vitro* germination of *L. polyphyllum* seeds collected from Himalayan subalpine coniferous forest, root emergence was enhanced (91%) by treatment with 100ppm IBA (25°C in 16/8h light/dark cycle) (Dhyani et al. 2014). Potassium nitrate has been used to promote germination, especially in darkness, of seeds with physiological dormancy (ISTA 2012; Hu et al. 2014). However, seeds of *L. polyphyllum* do not require light for germination, and the root emerges when seeds are buried in soil.

216 In the present study, a 30-minute treatment with NaOCl induced root emergence in
217 50.6% of the seeds within 56 days after seed sowing. However, only 15% of the seeds with
218 emerged roots had produced a shoot (epicotyl) after two growing seasons (A. Dhyani, pers.
219 obs.). The remaining seeds with emerged root did not produce a shoot and eventually decayed in
220 the styrofoam trays in the greenhouse. Thus, we can conclude that scarifying seeds with NaOCl
221 and incubating them in a low elevation greenhouse does not result in high percentages of
222 seedling production, probably because the temperature in the greenhouse was not low enough
223 (long enough) to break shoot dormancy. In contrast, in the laboratory the root emerged from
224 >90% of the seeds after 8 weeks of incubation in 12/12h light/dark cycle at 20°C. Then, after a
225 short period (14 days) of cold (0-4°C) moist treatment the shoot emerged from >80% of the
226 seeds moved from 0-4 to 20°C (Dhyani et al. 2013). Thus, a high percentage of the seeds give
227 rise to a healthy seedling in the laboratory under controlled temperature conditions, i.e. warm
228 for root production followed by cold for shoot dormancy break. On the other hand, only a low
229 percentage of seeds sown in a low elevation greenhouse produced a healthy seedling even when
230 seeds have been scarified with NaOCl or treated with GA₃, IBA, KNO₃ or thiourea. This
231 probably is due to lack of low temperatures to break shoot dormancy.

232 Following the protocol of Hong and Ellis (1996), we classify the seeds of *L. polyphyllum*
233 as having orthodox storage behavior. That is, seeds had high viability after being stored dry at -
234 20°C for 1 year. Thus, if seeds cannot be sown immediately after collection, they can be stored
235 dry at room temperature, 0-4°C or -20±1°C for 1 year without loss of viability. Barton (1936)
236 reported complete retention of viability in *L. auratum* seeds after 12 months in cold (1 or 5°C)
237 storage.

To produce seedlings, seeds collected in autumn can be stored dry at room temperatures until spring. Seeds sown in April in the northern temperate region will receive enough warm moist treatment for the root to emerge in June. Treatment with NaOCl, thiourea and potassium nitrate may decrease the time required for root emergence. However, seeds with an emerged root require at least 2 weeks of cold moist treatment (to break shoot dormancy) with shoot emergence in spring.

Acknowledgements

This work was supported by G. B. Pant Institute of Himalayan Environment and Development, Almora, Uttarakhand, India (grant number GBP/IERP/UA/04-05/12/305).

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- 1 **Table1** Percent viability and moisture content of seeds of *Lilium polphyllum* during one year of dry storage.

Temperature (°C)		Storage period (months)				
		0	3	6	9	12
10-35 (Room temperature)	MC(%)	12.8±1.8	11.9±0.5	10.1±1.4	7.6±1.1	6.8±0.8
	Viability %	100±0.0	100±0.0	100±0.0	97±4.2	95±3.8
0-4	MC(%)	12.8±1.8	11.5±1.3	10.7±0.8	9.6±1.8	8.8±1.3
	Viability %	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
-20±1	MC(%)	12.8±1.8	12.0±2.4	11.3±1.4	10.6±0.6	9.8±1.5
	Viability %	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0

- 2 **Note:** MC= Moisture Content

1
2 **Figure 1.**Effect of plant growth regulators, nitrogenous compounds and scarification on germination (root
3 emergence) of fresh seeds of *L. polyphyllum*. Different letters indicate significant differences ($P<0.05$) among all
4 the treatments ($n=3$).

5
6 **Figure 2.**Effect of plant growth regulators, nitrogenous compounds and scarification on MGT of fresh seeds of *L.*
7 *polyphyllum*. Different letters indicate significant differences ($P<0.05$) among all the treatments ($n=3$).
8

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Figure 1.

Figure 2.