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Requirements for seed germination and non-deep simple morphophysiological dormancy in *Sandersonia aurantiaca* (Colchicaceae)

Tetsuya KONDO[a]*, Yusuke KOKUMAI[b]

[a]Research Faculty of Agriculture, Hokkaido University, N9W9, Kita-ku, Sapporo, 060-8589, JAPAN, [b]School of Agriculture, Hokkaido University, N9W9, Kita-ku, Sapporo, 060-8589, JAPAN

Keywords: *Sandersonia aurantiaca*, seed germination, seed coat, light, morphophysiological dormancy

Abstract

Seed germination in *Sandersonia aurantiaca* is unstable and the requirements for germination are still unclear. We investigated requirements for germination using a broad range of treatments. The small embryos of *S. aurantiaca* seeds suggest morphophysiological dormancy (MPD), thus we also examined MPD levels in *S. aurantiaca* seeds. Radicles emerged from 18.8% of seeds without seed coat in light condition. In contrast, 95% of the seeds incubated in darkness produced radicles. In darkness, radicles emerged from only 1.3% of seeds with seed coat and from 60% of seeds without seed coat. In seeds without seed coat incubated in darkness, radicles emerged from 30% of seeds incubated at 30/20°C (alternating temperature) following a low temperature (cold stratification) at 5°C, but not from seeds at 35/25, 25, or 30°C following at 5 or 10°C. In seeds without seed coat incubated in darkness, regarding the effect of low temperatures (5°C and 10°C) before high temperatures, radicle emergence seemed to be higher in 5°C treatments than 10°C. These results indicate that *S. aurantiaca* seeds require removal of the seed coat, incubation in darkness, and low temperature to germinate. Radicles emerged, on average, from 40-70% of seeds without seed coat incubated in darkness at 5°C for 90 days followed by at 30/20°C. Furthermore, low temperature was required for embryo growth and radicle emergence at subsequent high temperature. Shoots emerged from more than 80% of the seeds with radicles at 30/20°C and at 20°C or more. These show that seeds of *S. aurantiaca* have non-deep simple morphophysiological dormancy.

*Corresponding author: Email: kondo@res.agr.hokudai.ac.jp

1. Introduction

Sandersonia aurantiaca Hook. is a monocotyledonous tuberous plant (Burge et al., 2008), belonging to a monotypic species in the Colchicaceae family (Sterling, 1975). Because this species was very common but recently become rare, it is protected by law in the Natal and Cape provinces, South Africa (Hennessy, 1977). Plant height is 50–100 cm (Burge et al., 2008), and they bloom lantern-shaped orange flowers in November and December; *S. aurantiaca* is commonly referred to as 'Christmas Bells' or 'Chinese lantern lily' (Finnie and Staden, 1996; Burge et al., 2008; Hennessy, 1977). *Sandersonia aurantiaca* was first cultivated in New Zealand in the 1980s for the production of cut flowers (Davies et al., 2002). Recently, Japan, Israel, and South Africa also produced significant quantities of these flowers (Burge et al., 2008). *Sandersonia aurantiaca* is propagated using tubers and seeds. Seed germination is unstable and can need two years (Warren, 1993). In New Zealand, for example, commercial growers leave the seeds in porous nets in a cold stream at mountain over winter to break seed dormancy (Finnie and Staden, 1996). Finnie and Staden (1996) showed that various treatments and their combination such as increased oxygen tension, scarification, stratification, and endosperm damage significantly increased seed germination to 66% within 2–3 weeks; however, the detailed methods and the applied experimental conditions were not described. Ijiro et al. (2004) observed that seeds planted outdoors in November in the Tochigi Prefecture, Japan, showed 46–72% shoot emergence in next May; the authors also showed that 25% of seeds germinated at 24/17°C by seed coat removal, shaking in a flask at 25°C for 30 days and planting at a depth of 0.5 cm. Zou et al. (2003) reported that scarification using sandpaper, nicking near the embryo and subsequent treatment with 300 mg/L gibberellic acid (GA3) at 20°C in darkness resulted in 69% germination.

From these previous studies, we surmise that scarification or removal of seed coat, exposure to low temperatures and GA3 application are potential factors affecting *S. aurantiaca* germination; however, the effects of seed coat removal, temperature, and light condition are still not comprehensively understood. In the present study, we conducted six experiments to investigate the effects of light, seed coat removal, and the temperature requirements on radicle emergence. We also investigated the optimal temperature for shoot emergence after radicle emergence.

The seeds of *S. aurantiaca* were reported to contain underdeveloped embryos (Finnie and Staden, 1996; Zou et al., 2001). Considering the underdeveloped embryos and the requirements for certain temperature treatments for germination, we hypothesize that seeds of *S. aurantiaca* exhibit morphophysiological dormancy (MPD). To determine the level of MPD, we investigated temperature requirements for embryo growth.

2. Materials and methods

2.1 *Experiments for radicle emergence*

2.1.1 *Seed treatments before experiments*

Six experiments were conducted to assess radicle emergence (Table 1). For these experiments, stems carrying fruits were harvested from the field in a farm in Oct. 2015, Oct. 2016, and Sep. 2017. Harvested stems were first stored in a greenhouse for 2–31 days and then stored on trays in the laboratory for 61–332 days depending on the experiment. Seeds were removed from the fruits, and immature seeds and debris were removed. Mature seeds were soaked in water in a beaker to facilitate removal of the seed coat. Because most of seeds repelled water and floated, Polysorbate 20 (Tween 20) or 99.5% ethanol was added under stirring until the seeds sunk. The water containing the seeds was stirred using a magnetic stirrer. The water was replaced once a day for five to eight days because its color changed to brown. Seed coats were then removed inside a metal-mesh tea strainer or an evaporating dish using a pestle. Seeds were sterilized using 1,000 ppm Benomyl solution under constant stirring using a magnetic stirrer for 24 h. Seeds stored in room, seeds soaked in water, and seeds removed seed coat were shown in Fig. 1.

2.1.2. *Sowing, light conditions, and observations*

In each experiment, four replicates of 20 seeds or 40 seeds were placed on three layers of filter paper moistened with distilled water in 9-cm diameter Petri dishes (Replicates number was shown in Table 1). Petri dish was sealed using parafilm (Pechiney Plastic Packaging, Menasha, Wisconsin, USA) to prevent evaporation. Seeds were incubated at either constant temperatures or alternating temperatures (high temperature for 12 h and low temperature for 12 h), and

Table 1. Seeds, pretreatments, replicates, and light condition in experiments for radicle emergence.

Expt.	Date offruits harvest	Days in green house	Days from storage in the laboratory to soaking seeds in water	Days for soaking in water (water was replaced every day)	Chemicals to sink seeds in water	Methods of seed coat removal	Chemical for sterilization (24h) after seed coat removal	Replicates, (number of seeds per Petri dish)	Light condition
1	2 Oct. 2015	12	68	5	Tween	Tea strainer	Benomyl	4, (20)	Light Darkness
2	2 Oct. 2015 12 Oct. 2015	12 2	168 158	6	Tween	Tea strainer None	Benomyl	4, (20)	Darkness
3	12 Oct. 2015	2	332	6	Tween	Tea strainer	Benomyl	4, (20)	Darkness
4	9 Oct. 2016	3	160	6	Ethanol	Evaporating dish	Benomyl	4, (20)	Darkness
5	15 Sep. 2017	31	61	8	Ethanol	Evaporating dish	Benomyl	4, (40)	Darkness
6	15 Sep. 2017	31	122	5	Tween Ethanol	Tea strainer	Benomyl	4, (40)	Darkness

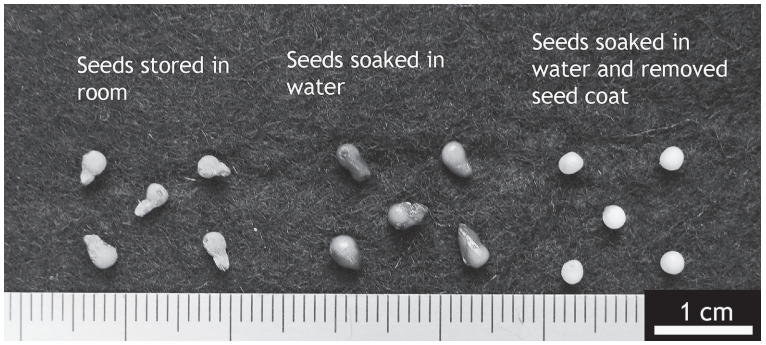

Fig. 1. Seeds stored in room, seeds soaked in water, and seeds removed seed coat.

either in light or darkness as shown in Tables 1–7. Seeds incubated at constant temperatures in light condition were irradiated using fluorescent light (ca. 25 μmol • m^{-2} • s^{-1}) for 12 h and kept in darkness for 12 h. Seeds incubated at alternating temperatures in light were exposed to light at high temperatures for 12 h and kept in darkness at low temperatures for 12 h. When seeds were incubated in darkness, Petri dishes were covered with two layers of aluminum foil. In cold stratification (0°C or 5°C), seeds were kept in darkness.

Seeds with an emerged radicle (≥ 2 mm) and rotten seeds were counted and removed every month. Therefore, even seeds kept in darkness were briefly exposed to light during the monthly observations. Moldy and soft seeds were considered as rotten. When mold was observed on seeds or on filter papers, they were removed from seeds using running water by gently rubbing without damaging seeds, and filter papers were replaced. Seeds were watered as needed. The completion of radicle and shoot emergence was considered as germination.

2.1.3 *Experiment 1: Effects of light on radicle emergence in seeds without seed coat*

Seeds harvested on 2 Oct. 2015 were pretreated and seed coat was removed as described above (hereafter, seeds without seed coat) (Table 1). Seeds without seed coat were incubated in 27 combinations of temperature/incubation period in light or in darkness, and a total of 54 treatments were used (Table 2); for instance, "0(90)→30/20 (60)→35/25 (60)" indicates that seeds were incubated at 0°C for 90 days and then at 30/20°C for 60 days followed by 60 days incubation at 35/25°C. Four replicates of 20 seeds each were used per treatment.

2.1.4 *Experiment 2: Effects of seed coat removal on radicle emergence*

Seeds were harvested on 2 Oct. and 12 Oct. in 2015, and pretreated as shown in Table 1. Seeds with seed coat (seeds that seed coat was not removed) or without seed coat in the two harvested date were incubated in darkness with three temperature treatments. Thus, a total 12 treatments were conducted (Table 3). Four replicates of 20 seeds each were used per treatment.

Table 2. Effects of light on radicle emergence (Experiment 1). Seeds without seed coat were used.

Treatment number			Radicle emergence (%)		Rotten (%)	
			Light	Darkness	Light	Darkness
Light	Darkness	Temperature treatments	Ave. ± STD	Ave. ± STD	Ave. ± STD	Ave. ± STD
TN1L	TN1D	0(90)→30/20(60)→35/25(60)	0.0 ± 0.0	3.8 ± 2.5	1.3 ± 2.5	0.0 ± 0.0
TN2L	TN2D	5(60)→30/20(60)→35/25(60)	8.8 ± 4.8	61.3 ± 14.4	5.0 ± 4.1	1.3 ± 2.5
TN3L	TN3D	5(90)→30/20(60)→35/25(60)	10.0 ± 7.1	30.0 ± 12.2	5.0 ± 7.1	2.5 ± 5.0
TN4L	TN4D	10(60)→30/20(60)→35/25(60)	10.0 ± 5.8	46.3 ± 10.3	1.3 ± 2.5	0.0 ± 0.0
TN5L	TN5D	10(90)→30/20(60)→35/25(60)	10.0 ± 7.1	47.5 ± 6.5	7.5 ± 15.0	0.0 ± 0.0
TN6L	TN6D	5(60)→30(90)	0.0 ± 0.0	0.0 ± 0.0	3.8 ± 4.8	0.0 ± 0.0
TN7L	TN7D	5(60)→25/15(60)→30/20(60)→35/25(60)	2.5 ± 5.0	22.5 ± 13.2	0.0 ± 0.0	0.0 ± 0.0
TN8L	TN8D	10(60)→30(90)	0.0 ± 0.0	0.0 ± 0.0	13.8 ± 24.3	0.0 ± 0.0
TN9L	TN9D	10(60)→25/15(60)→30/20(60)→35/25(60)	5.0 ± 4.1	25.0 ± 12.9	12.5 ± 21.8	1.3 ± 2.5
TN10L	TN10D	10(90)→30/20(60)→35/25(60)	12.5 ± 8.7	65.0 ± 16.8	0.0 ± 0.0	1.3 ± 2.5
TN11L	TN11D	5(60)→15/5(60)→30/20(60)→35/25(60)	0.0 ± 0.0	5.0 ± 7.1	0.0 ± 0.0	0.0 ± 0.0
TN12L	TN12D	5(60)→10(60)→25/15(60)→30/20(60)→35/25(60)	2.5 ± 2.9	10.0 ± 9.1	0.0 ± 0.0	0.0 ± 0.0
TN13L	TN13D	5(60)→15(60)→25/15(90)→30/20(60)→35/25(60)	1.3 ± 2.5	5.0 ± 10.0	0.0 ± 0.0	0.0 ± 0.0
TN14L	TN14D	5(90)→15(60)→30/20(60)→35/25(60)	1.3 ± 2.5	27.5 ± 14.4	0.0 ± 0.0	0.0 ± 0.0
TN15L	TN15D	10(60)→15/5(60)→30/20(60)→35/25(60)	2.5 ± 2.9	22.5 ± 9.6	0.0 ± 0.0	0.0 ± 0.0
TN16L	TN16D	10(60)→15(60)→25/15(60)→30/20(60)→35/25(60)	0.0 ± 0.0	1.3 ± 2.5	0.0 ± 0.0	0.0 ± 0.0
TN17L	TN17D	10(60)→20(60)→30/20(60)→35/25(60)	0.0 ± 0.0	2.5 ± 2.9	0.0 ± 0.0	0.0 ± 0.0
TN18L	TN18D	10(90)→20(60)→30/20(60)→35/25(60)	2.5 ± 2.9	10.0 ± 7.1	0.0 ± 0.0	0.0 ± 0.0
TN19L	TN19D	15/5(60)→5(60)→15/5(60)→30/20(60)→35/25(60)	3.8 ± 7.5	12.5 ± 5.0	0.0 ± 0.0	0.0 ± 0.0
TN20L	TN20D	15/5(60)→5(60)→15/5(60)→25/15(60)→30/20(60)→35/25(60)	2.5 ± 2.9	2.5 ± 5.0	1.3 ± 2.5	0.0 ± 0.0
TN21L	TN21D	20/10(60)→10(60)→20/10(60)→30/20(60)→35/25(60)	0.0 ± 0.0	22.5 ± 14.4	11.3 ± 22.5	1.3 ± 2.5
TN22L	TN22D	20/10(60)→10(60)→20/10(60)→25/15(60)→30/20(60)→35/25(60)	0.0 ± 0.0	12.5 ± 8.7	18.8 ± 37.5	2.5 ± 5.0
TN23L	TN23D	30/20(60)→15/5(60)→5(60)→15/5(60)→30/20(60)→35/25(60)	18.8 ± 11.1	55.0 ± 12.2	8.8 ± 17.5	0.0 ± 0.0
TN24L	TN24D	30/20(60)→20/10(60)→5(60)→20/10(60)→30/20(60)→35/25(60)	10.0 ± 7.1	73.8 ± 21.4	0.0 ± 0.0	1.3 ± 2.5
TN25L	TN25D	25/15(60)→15/5(60)→5(60)→15/5(60)→25/15(60)→30/20(60)→5/25(60)	0.0 ± 0.0	57.5 ± 15.5	18.8 ± 34.2	3.8 ± 7.5
TN26L	TN26D	30/20(60)→20/10(60)→10(60)→20/10(60)→30/20(60)→35/25(60)	16.3 ± 7.5	95.0 ± 4.1	3.8 ± 7.5	0.0 ± 0.0
TN27L	TN27D	25/15(60)→20/10(60)→10(60)→20/10(60)→25/15(60)→30/20(60)→35/25(60)	2.5 ± 2.9	17.5 ± 8.7	0.0 ± 0.0	0.0 ± 0.0

Table 3. Effects of seed coat removal on radicle emergence (Experiment 2). Seeds were incubated in darkness

Treatment number		Date of harvest	Temperature treatments	Radicle emergence (%)		Rotten (%)	
With seed coat	Without seed coat			With seed coat	Without seed coat	With seed coat	Without seed coat
				Ave. ± STD	Ave. ± STD	Ave .± STD	Ave. ± STD
TN28	TN31		0(60)→30/20(60)→35/25(60)	0.0 ± 0.0	2.5 ± 5.0	8.8 ± 10.3	16.3 ± 22.9
TN29	TN32	2 Oct. 2015	5(60)→30/20(60)→35/25(60)	0.0 ± 0.0	37.5 ± 9.6	8.8 ± 7.5	17.5 ± 18.5
TN30	TN33		10(60)→30/20(60)→35/25(60)	0.0 ± 0.0	18.8 ± 6.3	11.3 ± 2.5	8.8 ± 14.4
TN34	TN37		0(60)→30/20(60)→35/25(60)	0.0 ± 0.0	0.0 ± 0.0	56.3 ± 18.9	45.0 ± 35.4
TN35	TN38	12 Oct. 2015	5(60)→30/20(60)→35/25(60)	0.0 ± 0.0	60.0 ± 20.8	42.5 ± 28.4	22.5 ± 18.9
TN36	TN39		10(60)→30/20(60)→35/25(60)	1.3 ± 2.5	12.5 ± 10.4	80.0 ± 12.2	38.8 ± 26.9

2.1.5 *Experiment 3: Effects of alternating or constant high temperatures following low temperatures on radicle emergence*

Seeds were harvested on 12 Oct. 2015 and pretreated as shown in Table 1. Seeds without seed coat were incubated in darkness. To examine potential differences between alternating temperatures (30/20°C or 35/25°C) and constant temperatures (25°C or 30°C) following a low temperatures (5°C or 10°C) on radicle emergence, seeds were incubated with a total eight treatments (Table 4). Four replicates of 20 seeds each were used per treatment.

2.1.6 *Experiment 4: Effects of low temperatures (5°C or 10°C) followed by high temperatures on radicle emergence*

Seeds harvested on 9 Oct. 2016 were pretreated as shown in Table 1. Seeds without seed coat were incubated in darkness. Seven different settings of low temperature (5°C or 10°C) followed by a series of high temperatures were used (Table 5). Four replicates of 20 seeds each were used per treatment.

2.1.7 *Experiment 5:* Comparison between sequences of "Low→High temperature" and "High→Moderate→Low→Moderate→High temperature"

Seeds harvested on 15 Sep. 2017 were pretreated as shown in Table 1, and seed without seed coat were incubated in darkness. To identify whether the sequential temperature treatment "High (30/20°C)→Moderate (20/10°C)→Low (5°C or 10°C)→Moderate→High" would be more effective than a "Low→High" temperature treatment on radicle emergence, seeds were incubated under four regimes of "Low→High" temperatures and four sequential "High→Moderate→Low→Moderate→High" temperature regimes (Table 6). The temperature sequence used for TN98 was "High→Moderate→Low→ High"; however, the treatment was included in a "High→Moderate→Low→Moderate→High" sequence in this experiment. To obtain more accurate data, the temperature

Table 4. Effects of alternating or constant high temperatures following low temperatures on radicle emergence (Experiment 3). Seeds without seed coat were incubated in darkness.

Treatment number	Temperature treatments	Radicle emergence (%)	Rotten (%)
		Ave. ± STD	Ave. ± STD
TN56	5(60)→ 30/20(60)	30.0 ± 10.8	3.8 ± 7.5
TN57	5(60)→ 35/25(60)	0.0 ± 0.0	5.0 ± 7.1
TN61	10(60)→ 30/20(60)	13.8 ± 15.5	7.5 ± 8.7
TN62	10(60)→ 35/25(60)	0.0 ± 0.0	3.8 ± 7.5
TN58	5(60)→ 25(60)	0.0 ± 0.0	7.5 ± 9.6
TN59	5(60)→ 30(60)	0.0 ± 0.0	3.8 ± 4.8
TN63	10(60)→ 25(60)	0.0 ± 0.0	3.8 ± 2.5
TN64	10(60)→ 30(60)	0.0 ± 0.0	11.3 ± 13.1

Table 5. Effects of low temperatures (5°C or 10°C) followed by high temperatures on radicle emergence (Experiment 4). Seeds without seed coat were incubated in darkness. Significant difference between at 5°C and at 10°C in each set of low temperature was tested by t-test. There was a significant difference in root emergence between TN88 and TN81 in a set (*: $p < 0.05$).

Treatment number	Temperature treatments	Radicle emergence (%)	Rotten (%)
		Ave. ± STD	Ave. ± STD
TN86	5(90)→ 30/20(60)	68.8 ± 16.5	7.5 ± 9.6
TN79	10(90)→ 30/20(60)	65.0 ± 10.8	21.3 ± 4.8
TN87	5(90)→ 25/15(60)	57.5 ± 13.2	22.5 ± 24.0
TN80	10(90)→ 25/15(60)	53.8 ± 14.9	15.0 ± 20.4
TN88	5(90)→ 25/15(60)→ 30/20(60)	70.0 ± 4.1*	17.5 ± 5.0
TN81	10(90)→ 25/15(60)→ 30/20(60)	51.3 ± 11.1	8.8 ± 10.3
TN89	5(90)→ 20/10(60)→ 30/20(60)	58.8 ± 13.1	17.5 ± 6.5
TN82	10(90)→ 20/10(60)→ 30/20(60)	41.3 ± 10.3	13.8 ± 7.5
TN90	5(120)→ 30/20(60)	43.8 ± 19.7	43.8 ± 26.3
TN83	10(120)→ 30/20(60)	45.0 ± 17.3	48.8 ± 19.3
TN91	5(120)→ 25/15(90)→ 30/20(60)	62.5 ± 13.2	32.5 ± 18.5
TN84	10(120)→ 25/15(60)→ 30/20(60)	45.0 ± 21.2	11.3 ± 8.5
TN92	5(60)→ 20/10(60)→ 30/20(60)	51.3 ± 16.5	20.0 ± 10.8
TN85	10(60)→ 20/10(60)→ 30/20(60)	42.5 ± 16.6	28.8 ± 35.7

treatments “5(60)→30/20(60)” (TN94 and TN95), “10(60)→5(30)→30/20(60)” (TN96 and TN97), and “30/20(60)→20/10(60)→10(60)→20/10(60)→30/20(60)” (TN 99 and TN100) were conducted twice. Four replicates of 40 seeds each were used per treatment.

2.1.8 *Experiment 6: Effects of Tween or ethanol on radicle emergence*

Seeds harvested on 15 Sep. 2017 were pretreated as shown in Table 1, and seed without seed coat were incubated in darkness. Tween 20 or ethanol (98.8%) was added to the water in which seeds were soaked to prevent floating of the seeds. The seeds were subsequently incubated at each of the seven temperature treatments (Table 7). A total of 14 treatments was performed with four replicates of 40 seeds each per treatment.

2.2 *Required temperature for embryo growth*

Seeds harvested on 9 Oct. 2016 were pretreated as in Experiment 4 (Table 1). Ten seeds without seed coat were cut into thin sections along the longitudinal axis using an automicrotome. Embryo length and seed length were measured using an optical microscope equipped with a micrometer. The ratio of embryo length to seed length (hereafter referred to as E:S ratio) of each seed was recorded to produce an average value of each treatment. Forty seeds without seed coat each were sown on three layers of filter paper in two Petri dishes and incubated

Table 6. Comparison between sequences of "Low → High temperatures" and "High → Moderate → Low → Moderate → High temperatures" (Experiment 5). Seeds without seed coat were incubated in darkness. One-way ANOVA followed by Tukey's HSD test ($p < 0.05$) was used to compare percentages of radicle emergence among eight temperature treatments including sequence of "Low → High" temperatures and "High → Moderate → Low → Moderate → High" temperatures. There was no significant difference in percentages of radicle emergence among eight temperature treatments.

Pattern of temperature treatment	Treatment number	Temperature treatment	Radicle emergence (%)	Rotten (%)
			Ave. ± STD	Ave. ± STD
Low → High	TN94	5(60)→ 30/20(60)	51.3 ± 6.6	26.9 ± 5.5
	TN95	5(60)→ 30/20(60)	45.0 ± 9.1	35.6 ± 6.3
	TN96	10(60)→ 5(30)→ 30/20(60)	46.3 ± 6.3	44.4 ± 9.7
	TN97	10(60)→ 5(30)→ 30/20(60)	53.1 ± 4.3	30.6 ± 8.5
High → Moderate → Low → Moderate → High →	TN98	30/20(60)→ 20/10(60)→ 10(60) → 30/20(60)	31.9 ± 14.2	56.9 ± 13.9
	TN99	30/20(60)→ 20/10(60)→ 10(60) → 20/10(60)→ 30/20(60)	35.0 ± 17.6	60.0 ± 23.5
	TN100	30/20(60)→ 20/10(60)→ 10(60) → 20/10(60)→ 30/20(60)	53.1 ± 23.4	36.9 ± 9.0
	TN101	30/20(60)→ 20/10(60)→ 5(60) → 20/10(60)→ 30/20(60)	36.9 ± 17.1	53.8 ± 11.3

Table 7. Effects of Tween or ethanol on radicle emergence (Experiment 6). Seeds without seed coat were incubated in darkness. There was a significant differences only between TN115 (Tween) and TN123 (Ethanol) (t-test, ***: $p < 0.001$).

Treatment number		Temperature treatment	Radicle emergence (%)		Rotten (%)	
To sink seeds			Tween	Ethanol	Tween	Ethanol
Tween	Ethanol		Ave. ±STD	Ave. ±STD	Ave. ±STD	Ave. ±STD
TN110	TN118	5(60)→ 30/20(60)	80.0 ± 10.6	83.3 ± 8.3	12.5 ± 9.1	8.8 ± 7.8
TN111	TN119	5(90)→ 30/20(60)	84.4 ± 4.3	74.4 ± 8.8	8.8 ± 4.3	18.2 ± 7.7
TN112	TN120	5(90)→ 25/15(60)→ 30/20(60)	80.6 ± 7.7	80.9 ± 9.8	12.5 ± 4.6	17.9 ± 10.4
TN113	TN121	10(60)→ 30/20(60)	58.5 ± 24.2	73.8 ± 9.7	35.0 ± 21.9	21.3 ± 13.0
TN114	TN122	10(90)→ 30/20(60)	76.4 ± 15.2	53.1 ± 22.9	20.3 ± 12.6	41.9 ± 14.8
TN115	TN123	10(90)→ 25/15(60)→ 30/20(60)	83.1 ± 2.4***	47.5 ± 6.8	6.9 ± 6.9	40.6 ± 7.7
TN116	TN124	30/20(60)→ 20/10(60)→ 5(60)→ 20/10(60) → 30/20(60)	65.6 ± 18.8	31.3 ± 20.5	33.1 ± 19.6	65.0 ± 22.6

in "5(60)→30/20(30)" or "10(60)→30/20(30)" treatment in darkness. Ten seeds were taken out randomly from each treatment 60 and 90 days after the start of the incubation, and E:S ratios were calculated as described. Several seeds collected at 90 days showed 1–3-mm radicle emergence, therefore in these seeds, the length of the embryo inside seed was measured.

2.3 *Temperature requirements for shoot emergence after radicle emergence*

On 16 Sep. 2016, seeds with an emerged radicle (0.5–2.0 cm) were selected from the seeds used in Experiment 1. Twenty-five seeds were planted in a polyethylene container (15 x 10 x 5 cm with eight 5-mm diameter holes in the bottom) filled with red clay granule soil (about 5 mm in diameter) at a depth of 1 cm. A total four containers were placed on each one tray which was filled with water to a depth of 2 cm to allow the soil absorb water through the holes. Trays and containers were covered with transparent plastic sheets to prevent evaporation and kept at 20°C, 25°C, 30°C or 30/20°C. A light regime was maintained in 12 h fluorescent light and 12 h darkness. Water was added to the trays as required to maintain soil moisture. The numbers of shoots emerging from the soil were recorded every 2–3 days for 38 days until shoot emergence was almost completed.

2.4 *Assessment of seed coat permeability*

This experiment was conducted to determine whether the seed coat prevent water absorption. We used seeds collected on 15 Sept. 2017, stored in a greenhouse for one month and stored on a tray in the laboratory for 15 months. Eight lots of 50 seeds were weighed and then soaked in water under periodical stirring using a glass rod. Tween 20 was added until all seeds sink down. Water was replaced every day. After one week, seed coats of the seeds in four lots were removed by rubbing using a pestle in a metal-mesh tea strainer. The four replicates (lots) of 50 seeds with or without seed coat were dried using paper towels and then weighed. Seeds were then placed on filter papers in Petri dishes and kept at 30/20°C in darkness. Every two or three days for 31 days, the seeds were weighed after drying with paper towels.

2.5 *Statistical analyses*

The following analysis were carried out using the program SPSS Statistics 24.0 (IBM, New York. USA). To compare percentages of root emergence, *t*-tests were conducted when two treatments were involved, and one-way ANOVA followed by a Tukey's HSD test was conducted when three or more treatments were tested with one factor. Percentages were arcsine square-root transformed for statistical analyses. Statistical significance was reported at $p < 0.05$.

3. Results

3.1 *Experiment 1: Effects of light on radicle emergence in seeds without seed coat*

In all 27 temperature treatments, the percentages of radicle emergence were higher in seeds incubated in darkness than in light (Table 2). The maximum percentage of radicle emergence in light was 18.8% (TN23L); however, it was

95.0% in darkness (TN26D). The percentage of rotten seeds ranged from 0 to 18.8% over all treatments, and it appeared to be higher in light than in darkness. In treatments which included the temperature sequence "30/20(60)→35/25(60)", radicle emergence was almost consistently completed at 30/20°C (data not shown).

3.2 *Experiment 2: Effects of seed coat removal on radicle emergence*

In all treatments, the percentage of radicle emergence was higher in seeds without seed coat than with seed coat (Table 3). The maximum percentage of radicle emergence in seeds with seed coat was 1.3% (TN36), and 60.0% in without seed coat (TN38). The percentage of rotten seeds was higher in seeds collected on 12 Oct. 2015 than in those collected on 2 Oct. 2015, irrespective of the temperature treatment or presence of a seed coat.

3.3 *Experiment 3: Effects of alternating or constant high temperatures following low temperatures on radicle emergence*

Radicles emerged from seeds incubated only at 30/20°C following low temperature at 5°C (TN56, 30.0%) or at 10°C (TN61, 13.8%) (Table 4). No radicles emerged from seeds incubated at 35/25°C, 25°C, and 30°C following 5°C or 10°C incubation. The percentage of rotten seeds ranged from 3.8 to 11.3%.

3.4 *Experiment 4: Effects of low temperatures (5°C or 10°C) followed by high temperatures on radicle emergence*

In seven sets of two temperature treatments (5°C or 10°C), only one significant difference in radicle emergence was observed between TN88 and TN81 ($p < 0.05$; Table 5); however, the percentage of radicle emergence seemed to be higher in low-temperature treatments at 5°C than in those at 10°C. Radicles emerged from 41.3–70.0% of seeds, and the percentage of rotten seeds was 7.5–48.8%.

3.5 *Experiment 5: Comparison between sequences of "Low→High temperature" and "High→Moderate→Low→Moderate→High temperature"*

Differences in the percentages of radicle emergence were tested between eight treatments including the sequential temperatures "Low (5°C or 10°C)→High (30/20°C)" and "High (30/20°C) → Moderate (20/10°C)→Low (5°C or 10°C)→ Moderate (20/10°C)→High (30/20°C)" (Table 6). No significant difference was observed between the eight temperature treatments regarding the percentages of radicle emergence. However, the percentage of radicle emergence appeared to be higher in the "Low→High" treatment than in the sequence "High→Moderate →Low→Moderate→High", and the percentage of rotten seeds tended to be lower in the "Low→High" treatment than in the "High→Moderate→Low→Moderate→ High" sequence.

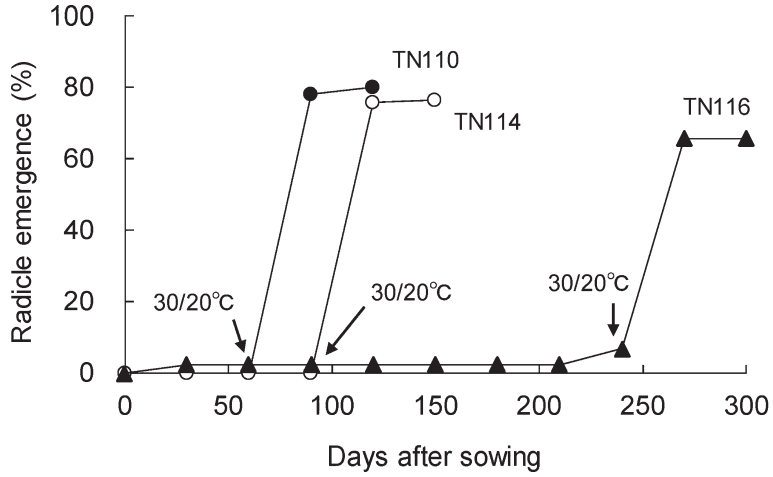

Fig. 2. The representative progress of radicle emergence in TN110, TN114, and TN116. TN110: 5(60)→30/20(60), TN114: 10(90)→30/20(60), TN116: 30/20(60)→20/10(60)→5(60)→20/10(60)→30/20(60). Most radicle emergence was completed within 30 days after transferred at 30/20°C

3.6 *Experiment 6: Effects of Tween 20 or ethanol on radicle emergence*

The statistical difference in root emergence was only observed between TN115 (treated with Tween 20) and TN123 (treated with ethanol) ($p < 0.001$; Table 7). The representative progress of radicle emergence in TN110, TN114, and TN116 was shown in Fig. 2. The major part of radicle emergence was completed within 30 days in 30/20°C incubation.

3.7 *Required temperature for embryo growth*

Initial embryo length was 2.3 mm, and the E:S ratio was 0.22 (Fig. 3). Embryos grew at 30/20°C following both low temperature treatments (5°C and 10°C).The E:S ratio increased to 0.44 by 30 days after seeds were transferred to 30/20°C, and radicles emerged from some seeds at this time.

3.8 *Temperature requirements for shoot emergence after radicle emergence*

Shoots began to emerge 5 to 7 days after planting (Fig. 4). Shoot emerged from 96 to 100% of the seeds after 30 days at 25°C, 30°C, and 30/20°C. Shoot emergence was delayed at 20°C; however, shoot emergence at 20°C eventually reached 84%.

3.9 *Assessment of seed coat permeability*

The weight of seeds with or without seed coat on day 0 (before soaking and seed coat removal) was shown in Fig. 5. The weight of 50 seeds with seed coat was 0.251 g on day 0, and that of 50 seeds without seed coat was 0.248 g. Seven

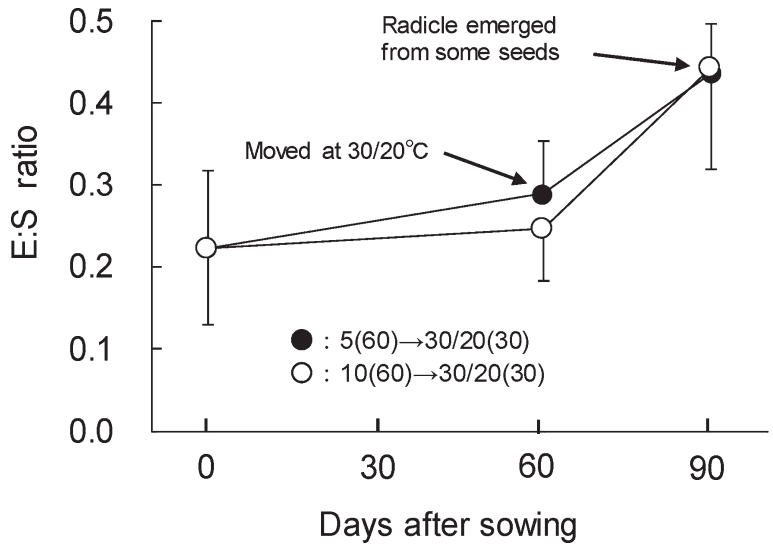

Fig. 3. Required temperature for embryo growth.
Vertical bars show + or - STD (n=10)

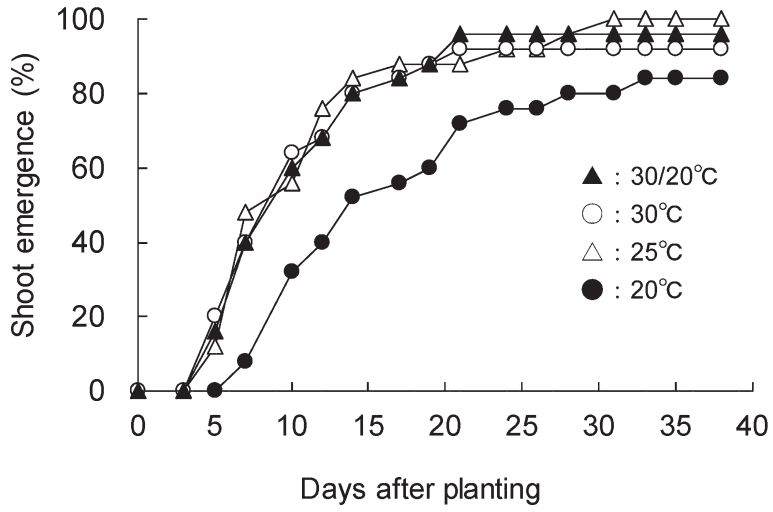

Fig. 4. Temperature requirements for shoot emergence after radicle emergence.

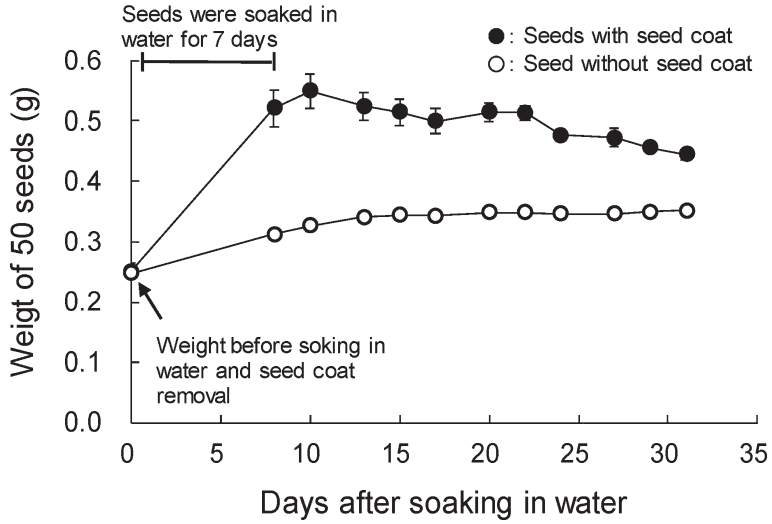

Fig. 5. Change of weight in seeds with or without seed coat after soaking in water and sowing.
The weight on 0 day indicates the weight before soaking in water and seed coat removal. Seeds were sown after 7days soaking, and seed coat was removed just before sowing. Four replicates of 50 seeds were measured. Vertical bars show STD (n=4).

days after soaking and after removal of the seed coat, the weight of seeds with seed coat increased to 0.521 g, and the weight of seeds without seed coat was 0.312 g. The weight of seeds without seed coat slightly increased to 0.352 g until the end of the experiment. In contrast, the weight of seeds with seed coat gradually decreased to 0.444 g until the end of the experiment.

4. Discussion

4.1 *Requirements for radicle and shoot emergence*

In *S. aurantiaca*, radicle emergence from seeds occurred substantially more frequent in darkness (Table 2) and after removal of the seed coat (Table 3). No difference in radicle emergence was observed between the Tween 20 and ethanol treatments (Table 7). Radicles emerged from seeds incubated at 30/20°C following low temperature treatment, but not from those incubated at other high temperatures (Table 4). Both 5°C and 10°C followed by 30/20°C was effective for radicle emergence (Table 5). There was no significant difference in radicle emergence between the temperature treatments "Low (5°C or 10°C)→High (30/20°C)" and "High→Moderate (20/10°C)→Low→Moderate→High" (Table 6). Radicle emergence was almost completed 30 days after the transfer to 30/20°C following low temperature treatments at 5°C or 10°C (Fig. 2). Taken together, radicle emergence averaged 40–70% when the seed coat was removed and seeds

were incubated in darkness under the sequential treatments "Low (5°C or 10°C)→High (30/20°C)" or "High (30/20°C)→Moderate (20/10°C)→Low (5°C or 10°C)→Moderate (20/10°C)→High (30/20°C)". However, the percentage of radicle emergence differed within the same or similar temperature treatments, depending on the experiment. For example, percentages of radicle emergence from seeds incubated under the treatment "5(90)→30/20(60)" were 68.8 and 84.4% in TN86 (Table 5) and TN111 (Table 7), respectively, but it was 30.0% under the treatment "5(90)→30/20(60)→35/25(60)" in TN3D (most radicle emergence was completed at 30/20°C before at 35/25°C) (Table 2). Similarly, radicle emergence from seeds incubated under a regime of "30/20(60)→20/10(60)→10(60)→20/10(60)→30/20(60)→35/25(60)" were 95.0% in TN26D (most radicle emergence was completed at 30/20°C before 35/25°C) (Table 2), but it was 53.1% under the regime "30/20(60)→20/10(60)→10(60)→20/10(60)→30/20(60)" in TN100 (Table 6). This suggests that some other unidentified factors increased radicle emergence to about 80% or more. The percentage of rotten seeds also varied between experiments. Because seed coats were removed manually, the extent of seed coat removal may have differed between experiments, and seeds may have been damaged during this procedure. Seed coat removal methods thus should be refined further. Shoots emerged from more than 80% of seeds with a radicle within 30 days of incubation at 30/20°C and at 20°C or more (Fig. 4).

Based on these results, to achieve 40–70% of radicle emergence within short time, seed coats should be removed and seeds should be kept at 5°C for 90 days and incubated at 30/20°C for 30 days in darkness. Seeds with an emerged radicle should be kept at 30/20°C to achieve shoot emergence (i.e. germination).

4.2 *Benefits of seed coat removal for radicle emergence*

Three effects of seed coat removal may be considered. First, the seed coat would prevent light penetration into the seed. However, radicles emerged from seeds without seed coat even in darkness and did not emerged under light (Table 2). Second, seeds have physical dormancy, thus they do not absorb water and do not germinate (Baskin and Baskin, 2014). The weight of seeds without seed coat increased slightly, whereas that of seeds with seed coat decreased gradually (Fig. 5). In seeds with seed coat, the seed coat was gradually removed during the experiment, therefore the seed weight decreased. As a consequence, the difference in water absorption by seeds with or without seed coat was unclear from this experiment. Third, some chemical compounds that occur in the seed coat or in fruits inhibit radicle emergence (Addicott and Lyon, 1969; Bhattacharya et al., 1999; Kim, 2019; Martínez-Honduvilla and Santos-Ruiz, 1978; Nakamura 1954; Ogawa and Iwabuchi, 2001; Witcombe et al., 1969; Xu et al., 2005). This factor was not investigated in the current study, however, we suggest that radicle emergence may have been inhibited by some chemical compounds in the seed coat.

4.3 *Dormancy level in S. aurantiaca seeds*

Seeds with MPD contain underdeveloped embryos, and embryos, radicles, and shoots are physiologically dormant, which must be terminated for germination (Baskin and Baskin, 2014). Levels of MPD are divided into two subclasses: (1) simple, in which the embryo grows at warm temperatures (≥ 15°C), (2) complex, in which the embryo grows at cold temperatures (about 0 to 10°C). Further, based on these cold and/or warm temperature requirements for embryo growth, root emergence, shoot emergence, and response to GA_3, nine levels of MPD have been shown (Baskin and Baskin, 2014). In seeds of *S. aurantiaca*, physiological dormancy of underdeveloped embryos and radicles was terminated at 5–10°C. After the low temperature treatment, embryos grew and radicles and shoots emerged at 30/20°C. Therefore, dormancy level in *S. aurantiaca* seeds can be classified as non-deep simple MPD (Baskin and Baskin, 2014). A previous study (Zou et al., 2003) showed that GA_3 at 20°C in the dark was effective for germination, indicating that GA_3 may substitute low temperature treatments.

5. Conclusions

The seed coat of *S. aurantiaca* should be removed, and seeds should be placed in the dark for germination to occur. Radicles emerged from 40–70% of seeds without seed coat when placed at 5°C for 90 days followed by 30/20°C in darkness. The reason for the higher percentage of radicle emergence following removal of the seed coat, however, remains to be elucidated. Considerable differences in percentages of radicle emergence and rotten seeds under the same or similar temperature treatments throughout experiments indicate that the factor affecting germination still need to be identified. Differences of the degree of seed coat removal may also account for the differences in germination percentages. Seeds of *S. aurantiaca* have an underdeveloped embryo. Both embryo growth and radicle emergence occurred at 30/20°C following low temperature at 5°C or 10°C. Shoots emerged from more than 80% of seeds with a radicle at 30/20°C or 20°C or more after 30 days. Therefore, the seeds of *S. aurantiaca* have non-deep simple MPD.

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