

# Protocol for Somatic Embryogenesis in Japanese Black Pine (*Pinus thunbergii* Parl.) and Japanese Red Pine (*Pinus densiflora* Sieb. et Zucc.)



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## 1 Introduction

Japanese black pine (*Pinus thunbergii* Parl.) and Japanese red pine (*P. densiflora* Zieb. et Zucc.) locally named “*kuromatsu*” and “*akamatsu*”, respectively, are two important forest tree species widely used for reforestation and landscaping in Japan. In addition, *P. thunbergii* is also planted along coastal areas to prevent sand movement, erosion, and damage by salt spray, and *P. densiflora* is also important as host species of the very expensive “*matsutake*” mushroom, *Tricholoma matsutake* (Kosaka et al. 2001; Maruyama and Hosoi 2016a).

However, in recent years, Japanese pine populations have further declined as a result of pine wilt disease, caused by the pinewood nematode, *Bursaphelenchus xylophilus* (Kuroda 2004; Akiba and Nakamura 2005). Pine wilt disease is one of the most serious pests in Japan, and has been a key critical factor in the mass mortality of the Japanese pine forests (Kanetani et al. 2001; Kanzaki et al. 2011; Maruyama and Hosoi 2014). The nematode is inferred to be native to North America and since its introduction into Japan at the beginning of the 20th century, the pinewood nematode has spread to Korea, Taiwan, and China and has devastated pine forests in East Asia (Togashi and Shigesada 2006). It was also found in Portugal in 1999 (Mota et al. 1999).

Somatic embryogenesis is one of the most promising techniques for mass propagation of selected trees. It allows, the ex situ conservation of genetic resources by cryopreservation techniques, and for the purposes of genetic transformation (Park et al. 1998; Bonga 2016; Maruyama and Hosoi 2016b). Studies on somatic embryogenesis of Japanese pines has been reported (Ishii et al. 2001; Taniguchi 2001; Hosoi and Ishii 2001; Maruyama et al. 2005a, b, 2007; Shoji et al. 2006;

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Hosoi and Maruyama 2012; Kim and Moon 2014). In these studies, the low induction frequency of embryonal masses reported, reflect the arduousness in establishing embryogenic cultures in comparison with other Japanese conifers (Ogita et al. 1999; Maruyama et al. 2000, 2002, 2005c; Taniguchi and Kondo 2000; Igasaki et al. 2003; Taniguchi et al. 2004; Nakagawa et al. 2011; Maruyama and Hosoi 2012a; Hosoi and Maruyama 2016). In addition, although high somatic embryo maturation frequencies were observed in maturation media supplemented with polyethylene glycol, the achieved germination frequencies were relatively low (Maruyama et al. 2005a, b). Later, an improved protocol for somatic embryo germination of Japanese pines based on the desiccation of somatic embryos after the maturation on medium containing polyethylene glycol was reported (Maruyama and Hosoi 2012b). This post-maturation treatment markedly increased germination frequencies and considerably improved synchronization during the germination period similarly reported in other conifers (Roberts et al. 1990, 1991; Hay and Charest 1999; Klimaszewska and Cyr 2002; Stasolla and Yeung 2003; Klimaszewska et al. 2007).

The protocol described here is based on somatic embryogenesis initiated from immature seeds and plant regeneration obtained from somatic embryos after maturation on medium with polyethylene glycol. Procedures including explant preparation, embryonal mass induction and proliferation, somatic embryo maturation, plant conversion, and acclimatization are described.

## **2 Protocol for Somatic Embryogenesis in Japanese Black Pine and Japanese Red Pine**

### ***2.1 Culture Media***

1. The culture media used for Japanese black pine and Japanese red pine somatic embryogenesis are described in Table 1.
2. Note that this protocol consists of several in vitro culture stages differing in medium, culture condition, and duration as described in Table 2.
3. Adjust medium to pH 5.8, and autoclave for 15 min at 121 °C and 1.1 kg cm<sup>-2</sup>.
4. Amino acids stock solutions and abscisic acid (ABA) are filter sterilized and added to the medium after autoclaving.
5. Dispense media in culture vessels as specified in Table 2. Plates are sealed with Parafilm (Parafilm M<sup>®</sup> film, Bemis Company, Inc., Wisconsin, USA). Flasks are capped with transparent Tetoron film (Toray Ind., Tokyo, Japan).

**Table 1** Constituents of culture media<sup>a</sup> for Japanese black pine and Japanese red pine plant regeneration system via somatic embryogenesis

Constituents	M1 (mg/L)	M2 (mg/L)	M3 (mg/L)	M4 (mg/L)	M5 (mg/L)
<i>Basal salts</i>					
KNO <sub>3</sub>	500	500	1000	500	500
NaNO <sub>3</sub>	30	30	60	30	30
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	80	80	160	80	80
KH <sub>2</sub> PO <sub>4</sub>	35	35	70	35	35
MgSO <sub>4</sub> ·7H <sub>2</sub> O	250	250	500	250	250
CaCl <sub>2</sub> ·2H <sub>2</sub> O	37.5	37.5	75	37.5	37.5
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	30	30	60	30	30
KCl	40	40	750	40	40
MnSO <sub>4</sub> ·4H <sub>2</sub> O	10	10	20	10	10
H <sub>3</sub> BO <sub>3</sub>	20	20	40	20	20
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	12.5	12.5	25	12.5	12.5
KI	0.5	0.5	1	0.5	0.5
CuSO <sub>4</sub> ·5H <sub>2</sub> O	1.2	1.2	2.4	1.2	1.2
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.1	0.1	0.2	0.1	0.1
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.1	0.1	0.2	0.1	0.1
FeSO <sub>4</sub> ·7H <sub>2</sub> O	15	15	30	15	15
Na <sub>2</sub> -EDTA	20	20	40	20	20
<i>Vitamins</i>					
Myo-Inositol	500	500	1000	500	500
Thiamine hydrochloride	2.5	2.5	5	2.5	2.5
Pyridoxine hydrochloride	0.25	0.25	0.5	0.25	0.25
Nicotinic acid	2.5	2.5	5	2.5	2.5
Glycine	2.5	2.5	5	2.5	2.5
<i>Plant growth regulators</i>					
2,4-D	2.21	0.663			
BAP	1.125	0.225			
ABA			26.4		
<i>Other additives</i>					
Casein acid hydrolysate	500				
Glutamine	1000	1500	7300	400	
Asparagine			2100		
Arginine			700	250	
Citrulline			79		
Ornithine			76		
Lysine			55		
Alanine			40		
Proline			35	100	
Sucrose	10,000	30,000			30,000

(continued)

**Table 1** (continued)

Constituents	M1 (mg/L)	M2 (mg/L)	M3 (mg/L)	M4 (mg/L)	M5 (mg/L)
Glucose				30,000	
Maltose			50,000		
Polyethylene glycol			100,000		
Activated charcoal			2000	2000	5000
Gellan gum	3000	3000	3000	6000	
Agar					11,500
pH	5.8	5.8	5.8	5.8	5.8

<sup>a</sup>M1 (Embryogenic culture initiation medium), M2 (Embryonal mass maintenance/proliferation medium), M3 (Somatic embryo maturation medium), M4 (Somatic embryo germination medium), M5 (Somatic plant growth medium)

## 2.2 *Explant Preparation*

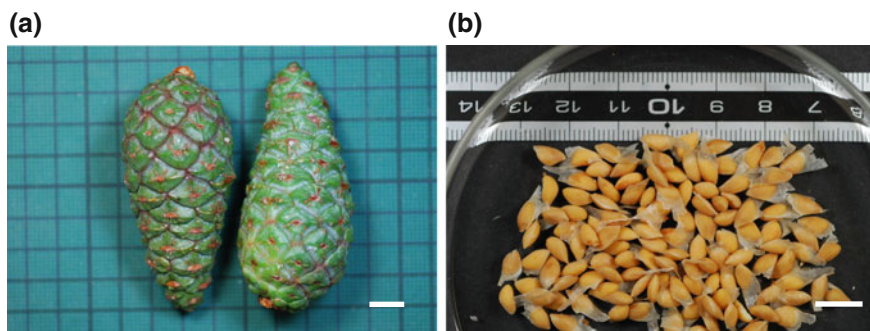
1. Collect immature cones (Fig. 1a) from mother trees in mid-July.
2. Remove the seeds from the immature cones.
3. Disinfect excised seeds (Fig. 1b) with 1% (w/v available chlorine) sodium hypochlorite solution for 15 min and then rinse five times with sterile distilled water, 3 min each time.
4. Transfer the sterile seeds in a sterile plate.
5. Remove the seed coat with sterile scalpel and forceps and aseptically isolate megagametophytes from the seeds under a dissecting microscope.

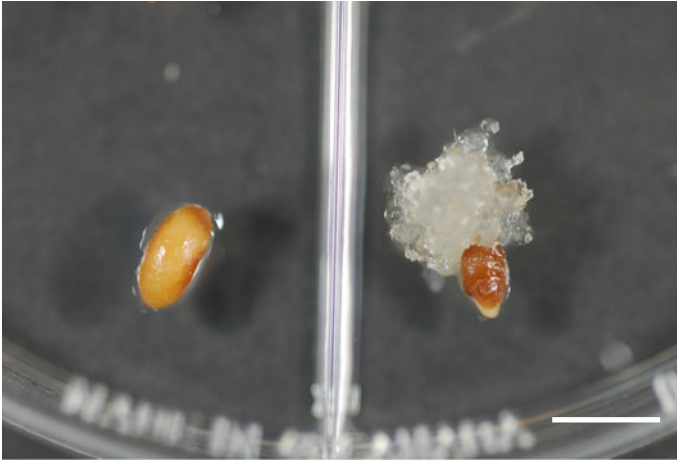
## 2.3 *Embryogenic Culture Initiation*

1. For induction of embryonal masses, put horizontally the isolated megagametophyte explants on the surface of initiation medium (M1, Table 1) contained in Quad-plates (three explant per well, twelve per plate).
2. Seal culture plates with Parafilm and incubate under conditions described in Table 2.
3. The presence (Fig. 2) or absence of distinct early stages of somatic embryos characterized by an embryonal head (dense cells) with suspensor system (elongated cells) from the explant is observed weekly under the inverted microscope, up to 3 months. Initiation of embryonal masses is recorded if distinct early stages of somatic embryos proliferated after the first subculture.

**Table 2** Medium, culture conditions, and culture durations for each stage of somatic embryogenesis in Japanese black pine and Japanese red pine

Stage	Medium <sup>a</sup>	Culture conditions <sup>b</sup>	Duration (week)
1. Embryogenic culture initiation	M1	Dark, 90 × 15 mm quad-plates (30–35 ml medium/plate) 3 megagametophytes/well (12/plate)	4–12
2. Embryonal mass maintenance/proliferation	M2 or M2 <sup>c</sup>	Dark, 90 × 15 mm quad-plates (30–35 ml medium/plate) 12 embryonal masses/plate Dark, 100 ml flasks, 60–80 rpm (50 ml medium/flask)	2–3 1–2
3. Somatic embryo maturation	M3	Dark, 90 × 20 mm mono-plates (30–40 ml medium/plate) 5 embryonal masses/plate	6–8
4. Somatic embryo desiccation	Filter paper	Dark, six-well multiplates (20–30 somatic embryos/well)	2–3
5. Somatic embryo germination	M4	Light, 90 × 20 mm mono-plates (30–40 ml medium/plate)	2–4
6. In vitro growth of somatic plants	M5 or Vermiculite	Light, 300 ml flasks (100 ml medium/flask) or (100 ml vermiculite/flask irrigated with a 0.1% (v/v) hyponex solution <sup>d</sup> )	8–12
7. Ex vitro acclimatization of somatic plants	Vermiculite or Kanuma soil	Light, plants inside a growth chamber at 25/20 ± 1 °C and 80 ± 5% relative humidity were irrigated with a 0.1% (v/v) Hyponex solution <sup>d</sup>	3–5

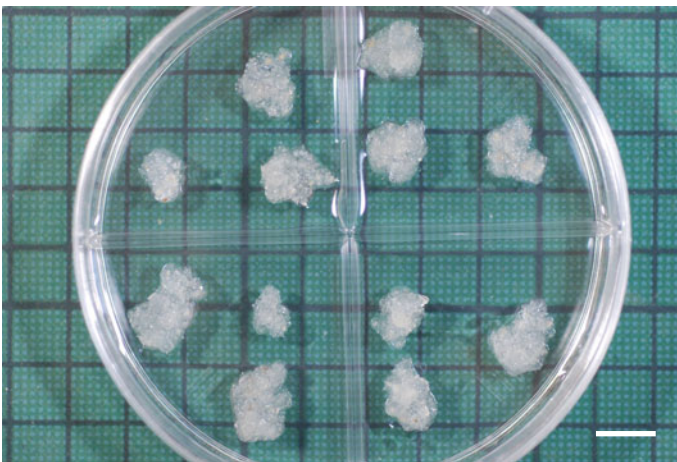
<sup>a</sup>See Table 1<sup>b</sup>Culture at 16-h photoperiod (65 μmol m<sup>-2</sup> s<sup>-1</sup>) or darkness at 25 ± 1 °C<sup>c</sup>M2 medium without gellan gum<sup>d</sup>Hyponex 6-10-5 plant-food solution (The Hyponex Co., Inc., Hyponex Japan, Osaka, Japan)**Fig. 1** **a** Immature cones of Japanese black pine, **b** excised seeds from the cones. Bars 1 cm



**Fig. 2** Induction of embryonal mass from megagametophyte explant of Japanese black pine. *Bar:* 1 cm

#### ***2.4 Maintenance and Proliferation of Embryonal Masses***

1. Collect embryonal masses from initiation medium with forceps and transfer to maintenance/proliferation medium (M2, Table 1). Seal plates with Parafilm and culture as describe in Table 2.
2. For subsequently maintenance/proliferation routines (Fig. 3), transfer twelve embryonal masses (about 20 mg FW each) onto M2 medium (three masses per well, twelve per plate) and subculture at 2 to 3-week intervals.

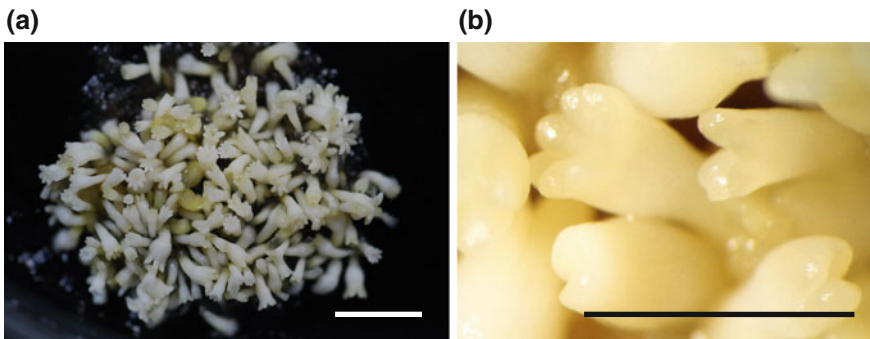


**Fig. 3** Maintenance and proliferation of embryonal masses of Japanese black pine. *Bar:* 1 cm

3. As an alternative method for more rapid proliferation making of cell suspension cultures, transfer embryonal masses (about 50 mg FW) to 100 ml flasks containing 50 ml liquid medium (M2 medium without gellan gum) and culture in rotary shaker with shaking at 60–80 rpm in darkness at  $25 \pm 1$  °C. For continuously proliferation routines in liquid medium, subculture embryonal masses to same fresh medium (about 0.5 ml suspension culture in 50 ml fresh medium) at 1 to 2-week intervals.

## 2.5 Maturation of Somatic Embryos

1. Collect proliferated embryonal masses from maintenance/proliferation medium with forceps and transfer five masses (about 100 mg FW each) onto plate containing maturation medium (M3, Table 1). Homogeneously disperse each embryonal mass with forceps on a surface equivalent to a circle of about 2.5–3 cm in diameter. Seal plates with Parafilm and culture as describe in Table 2.
2. For suspension cultures, collect embryonal masses on 100  $\mu$ m nylon screen and rinse embryonal masses with M2 medium without plant growth regulators and gellan gum. Resuspend embryonal masses in same medium (about 500 mg FW in 2.5–3 ml medium) and homogeneously disperse with pipettes onto 70-mm-diameter filter paper disks over each plate containing maturation medium (M3, Table 1). Seal plates with Parafilm and culture as describe in Table 2.
3. Initial formation of cotyledonary embryos are observed about 4 week after transfer of embryonal masses to the maturation medium, and is evident at 6–8 week of culture (Fig. 4).



**Fig. 4** Cotyledonary embryo formation in Japanese black pine (a) and Japanese red pine (b). Bars 1 cm

## 2.6 *Somatic Embryo Desiccation*

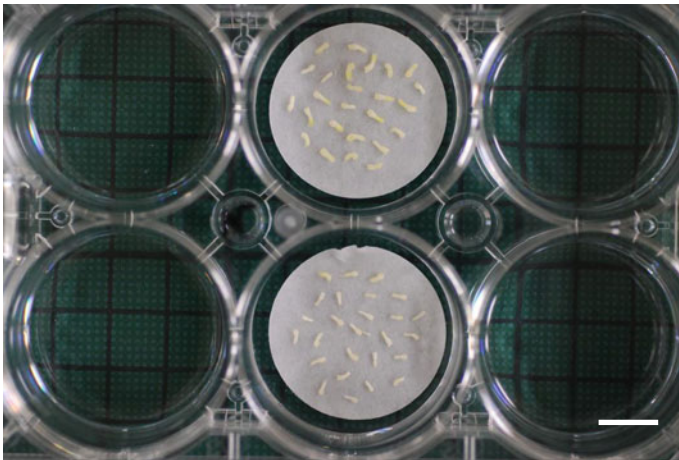
1. Collect cotyledonary embryos from maturation medium and transfer onto 30-mm-diameter filter paper disks over plate containing germination medium (M4, Table 1).
2. Transfer the embryos placed over 30-mm-diameter filter paper disks into 2 (central) wells of a Six-well multiplate in which the remaining 4 (side) wells are filled with about 5 ml of sterile water (Fig. 5).
3. Seal plates with Parafilm and incubate as describe in Table 2.

## 2.7 *Germination of Somatic Embryos*

1. Collect filter paper disks containing desiccated somatic embryos and transfer onto plates containing germination medium (M4, Table 1).
2. Seal plates with Parafilm and culture as describe in Table 2.
3. About 2–4 week after transfer to germination medium germinated embryos (Fig. 6) can be transferred to growth medium for plant conversion.

## 2.8 *In Vitro Growth of Somatic Plants*

1. Collect germinated somatic embryos from germination medium and transfer to flasks containing growth medium (M5, Table 1; Fig. 7a). Flasks are capped with transparent Teton film and culture as described in Table 2.

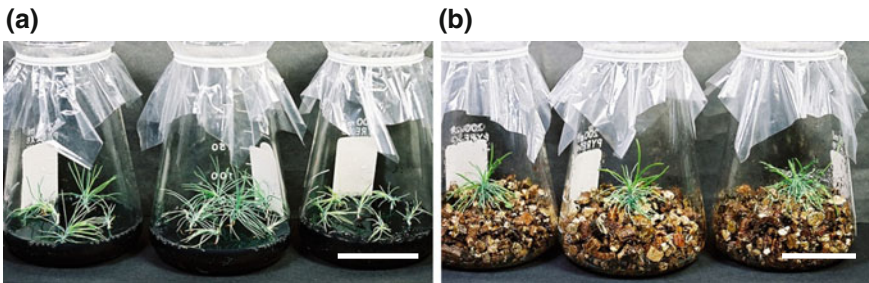


**Fig. 5** Desiccation of somatic embryos in six-well multiplate at high relative humidity. Bar 1 cm





**Fig. 6** Germination of somatic embryos after desiccation treatment. *Bar* 1 cm

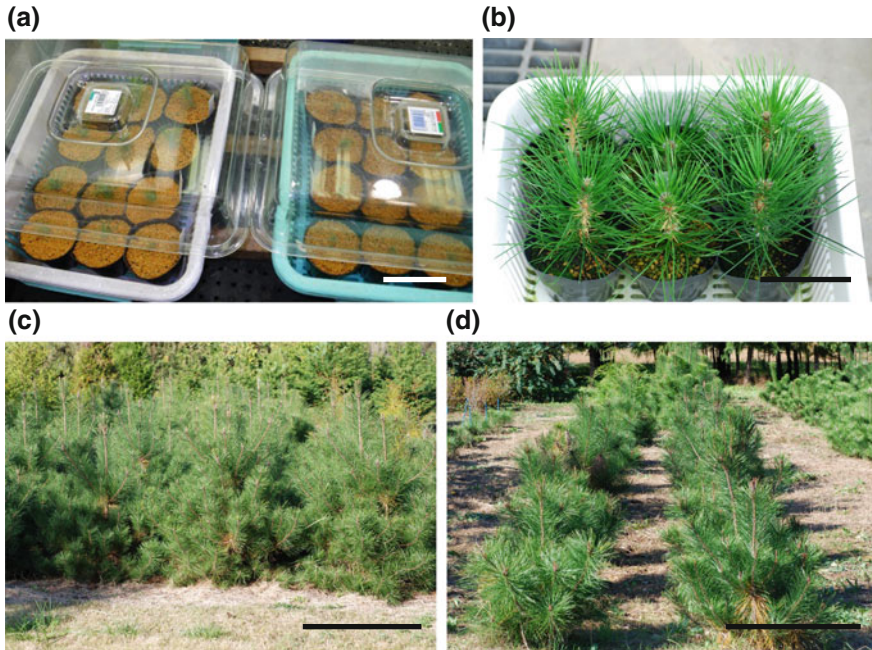


**Fig. 7** In vitro growth of somatic plants after transfer to flasks containing M5 medium (a) or Vermiculite with Hyponex nutrient solution (b). *Bars* 5 cm

2. As an alternative method, transfer germinated somatic embryos to flasks containing Vermiculite with Hyponex nutrient solution and culture as described in Table 2 (Fig. 7b).
3. About 8–12 week after transfer to growth medium somatic plants can be transferred to ex vitro conditions for acclimatization.

### 2.9 Ex Vitro Acclimatization and Field Transfer

1. Remove somatic plants from culture flasks and transplant into plastic pots filled with vermiculite or Kanuma soil.



**Fig. 8** Acclimatization and field transfer of somatic plants. **a** Acclimatization in plastic boxes, **b** acclimatized plants growing in a greenhouse before transplanting to the field, **c**, **d** somatic plants of Japanese black pine and Japanese red pine growing in the field. Bars **a**, **b** 10 cm, **c**, **d** 1 m

2. For the first 2 week somatic plants are kept under high relative humidity inside plastic boxes with transparent covers. During the first 2 weeks irrigate only with water as needed.
3. After the first 2 week, open the covers gradually and irrigate the pots with Hyponex nutrient solution as described in Table 2 (Fig. 8a).
4. Remove covers completely 4 wk after transplanting.
5. Best acclimatization and growth of somatic plants are recorded keeping the pots inside the growth chamber (80% relative humidity, and alternating temperature of 25 °C for 16-h photoperiod and 20 °C for 8-h darkness).
6. Subsequently, transfer the acclimatized plants to a greenhouse until they reach an approximate height of 15–20 cm (Fig. 8b) to be transplanting to the field preferably in spring season.
7. Remove somatic plants from pots and transplant to the permanent field location (Fig. 8c, d).

### 3 Conclusions and Future Prospects

An improved propagation system has been achieved for Japanese black pine and Japanese red pine with described protocol based on somatic embryogenesis initiated from immature seeds and plant regeneration obtained from somatic embryos after maturation on medium with polyethylene glycol. Post-maturation treatment based on the desiccation of somatic embryos after polyethylene glycol-mediated maturation markedly increased germination frequencies and synchronization during the germination period (Maruyama and Hosoi 2012b). However, further efforts are needed to establish an optimal protocol for the commercial production. Protocol modifications to increase the induction frequency of embryonal masses, as well as to develop efficient bioreactors that can be used for the large-scale production of somatic embryos are necessary. On the other hand, since most tree breeding programs have adopted a system of recurrent selection, strategies using vegetative propagation have additional advantages over traditionally improved seeds (Park 2002). Although at present, embryogenic systems derived from vegetative explants of mature pines have been reported in a few species (Texeira da Silva and Malabadi 2012), the positive results on somatic embryogenesis induction attributed to the reported methods have largely proven unrepeatable with other pine species (Trontin et al. 2016). For a more efficient implementation of somatic embryogenesis in tree breeding programs, more efforts are needed to develop a methodology to control the initiation of somatic embryogenesis from adult vegetative explants of Japanese pines.

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