REPUBLIC OF SOUTH AFRICA

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PATENTS ACT, 1978

CERTIFICATE

In accordance with section 44 (1) of the Patents Act, No. 57 of 1978, it is hereby certified that:

YANBIAN UNIVERSITY

Has been granted a patent in respect of an invention described and claimed in complete specification deposited at the Patent Office under the number

2022/07141

A copy of the complete specification is annexed, together with the relevant Form P2.

In testimony thereof, the seal of the Patent Office has been affixed at Pretoria with effect from the **28th** day of **September 2022**

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54	Title of invention
MEDIUM AND METHOD FOR TISSUE CULTURE OF ACER PSEUDO-SIEBOLDIANUM	

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COMPLETE SPECIFICATION
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TITLE OF INVENTION

54	MEDIUM AND METHOD FOR TISSUE CULTURE OF ACER PSEUDO-SIEBOLDIANUM

MEDIUM AND METHOD FOR TISSUE CULTURE OF *ACER PSEUDO-SIEBOLDIANUM*

TECHNICAL FIELD

[01] The present invention relates to the technical field of tissue culture, in particular to a medium and method for tissue culture of *Acer pseudo-sieboldianum*.

BACKGROUND ART

[02] Maples are a world-famous color-leaved tree species, with more than 200 species in the world and more than 160 species in China, mainly growing in the Yangtze River Basin and provinces to the south of the Yangtze River Basin. Nearly 10 species are native to Northeast China, but most of them are in the wild. *Acer pseudo-sieboldianum (Pax) Komarov* (*Acer pseudo-sieboldianum* for short) is a acer woody color-leaved tree species of Aceraceae. As its tree posture, leaf shape and wing fruit have high ornamentality, *Acer pseudo-sieboldianum* is a rare, excellent ornamental tree species for gardening and coloring in the north. At present, *Acer pseudo-sieboldianum* is mainly propagated by seeds, but the progeny of seed propagation are highly variable and susceptible to seasons, which cannot satisfy the needs of modern landscaping.

[03] Tissue culture and seedling technologies not only maintain the purity and characteristics of the species, but also increase the speed of propagation and survival rate. However, tissue culture of woody plants is difficult. There are more than 200 species of maples in the world, and there are more than 160 species in China. A relatively complete tissue culture regeneration system has been established for only a few species, such as red maple, *Acer davidii*, *Acer saccharum Marshall*, *Acer ginnala Maxim*, *Acer rubrum Red Maple* and *Acer negundo Linn*. However, the tissue culture propagation method of *Acer pseudo-sieboldianum* with high ornamental value, which is unique to Northeast China, has never been reported.

SUMMARY

[04] In view of this, the present invention provides a medium and method for tissue culture of *Acer pseudo-sieboldianum*. The medium formula can remarkably decrease the contamination rate of *Acer pseudo-sieboldianum* explants, promote the growth of axillary buds, increase the proliferation coefficient and rooting rate. *Acer pseudo-sieboldianum* with excellent traits can be rapidly propagated by using the plant tissue culture method.

[05] In order to realize the above-mentioned objective, the present invention adopts the following technical solutions:

[06] The present invention provides a medium for tissue culture of *Acer pseudo-sieboldianum*, including a priming medium, a proliferation medium and a rooting medium, wherein

[07] the priming medium is a 1/2MS medium containing 0.05 - 0.25 mg/L IBA and 25 - 35 g/L sucrose;

[08] the proliferation medium is a MS medium containing 0.05 - 0.20 mg/L IBA, 0.5

- 1.5 mg/L CPPU and 25 - 35 g/L sucrose; and

[09] the rooting medium is a 1/2MS medium containing 0.10 - 0.40 mg/L IBA and 15 - 25 g/L sucrose.

[10] Preferably, the priming medium is a 1/2MS medium containing 0.05 - 0.15 mg/L IBA and 28 - 32 g/L sucrose;

[11] the proliferation medium is a MS medium containing 0.05 - 0.15 mg/L IBA, 0.8 - 1.2 mg/L CPPU and 28 - 32 g/L sucrose; and

[12] the rooting medium is a 1/2MS medium containing 0.15 - 0.25 mg/L IBA and 18 - 22 g/L sucrose.

[13] Preferably, the priming medium is a 1/2MS medium containing 0.10 mg/L IBA and 30 g/L sucrose;

[14] the proliferation medium is a MS medium containing 0.10 mg/L IBA, 1.0 mg/L CPPU and 30 g/L sucrose; and

[15] the rooting medium is a 1/2MS medium containing 0.20 mg/L IBA and 20 g/L sucrose.

[16] The present invention provides a method for tissue culture of *Acer pseudo-sieboldianum*, including the following steps:

[17] taking and pretreating current-year branches of *Acer pseudo-sieboldianum* to obtain explants;

[18] sterilizing the explants;

[19] inoculating the sterilized explants into a priming medium for primary culture, wherein the priming medium is a 1/2MS medium containing 0.05 - 0.25 mg/L IBA and 25 - 35 g/L sucrose;

[20] transferring axillary buds after primary culture to a proliferation medium for proliferation culture, wherein the proliferation medium is a MS medium containing 0.05 - 0.20 mg/L IBA, 0.5 - 1.5 mg/L CPPU and 25 - 35g/L sucrose; and

[21] transferring cluster seedlings after proliferation culture to a rooting medium for rooting culture, wherein the rooting medium is a 1/2MS medium containing 0.10 - 0.40 mg/L IBA and 15 - 25 g/L sucrose.

[22] In the present invention, the pretreatment is as follows: the leaves of the current-year branches are removed, and the branches are divided into 1.5 - 2.0 cm stems with axillary buds, and washed.

[23] Explant contamination is one of the three major problems in plant tissue culture technology, especially in woody plants, where high contamination rates are often caused by incomplete disinfection of explants or endophytes, thus affecting the rapid propagation of woody plants. In the present invention, by the secondary disinfection method, the contamination rate in the priming medium of *Acer pseudo-sieboldianum* is decreased from 70 - 80% to 30 - 40%, the efficiency of obtaining tissue culture seedlings is improved, the production cost is reduced, the working time is saved and an effective guarantee is provided for further obtaining regenerated plants.

[24] As a preference, the sterilization is as follows: the explants are treated with sodium hypochlorite for 20 - 40 min after washing, sealed in sterilized culture flasks, placed at 2 - 8℃ for 20 - 30 h; after that, the explants are treated with alcohol for 20 - 40 s, then disinfected with mercuric chloride for 20 - 40 min, and finally rinsed with

sterile water.

[25] Preferably, the sterilization is as follows: the explants are treated with sodium hypochlorite for 30 min, then washed, sealed in sterilized culture flasks, and placed at 4℃ for 24 h; after that, the explants are treated with alcohol for 30 s, then disinfected with mercuric chloride for 30 min, and finally rinsed with sterile water.

[26] As a preference, a mass volume percentage of the sodium hypochlorite is 3% - 7%.

[27] As a preference, a volume percentage of the alcohol is 60% - 80%.

[28] As a preference, a mass volume percentage of the mercuric chloride is 0.08% - 0.12%.

[29] Preferably, the mass volume percentage of the sodium hypochlorite is 5%.

[30] Preferably, the volume percentage of the alcohol is 70%.

[31] Preferably, the mass volume percentage of the mercuric chloride is 0.1%.

[32] As a preference, a temperature of the primary culture is 23 - 25℃, an illumination time is 12 h/d, and an illumination intensity is 2,000 Lx.

[33] As a preference, a temperature of the proliferation culture is 23 - 25℃, an illumination time is 12 h/d, and an illumination intensity is 2,000 Lx.

[34] As a preference, a temperature of the rooting culture is 23 - 25℃, an illumination time is 12h/d, and an illumination intensity is 2,000 Lx.

[35] The present invention provides a medium and method for tissue culture of *Acer pseudo-sieboldianum*. The medium includes a priming medium, a proliferation medium and a rooting medium, wherein the priming medium is a 1/2MS medium containing 0.05 - 0.25 mg/L IBA and 25 - 35 g/L sucrose; the proliferation medium is a MS medium containing 0.05 - 0.20 mg/L IBA, 0.5 - 1.5 mg/L CPPU and 25 - 35 g/L sucrose; and the rooting medium is a 1/2MS medium containing 0.10 - 0.40 mg/L IBA and 15 - 25 g/L sucrose. The prevent invention has the following technical effects:

[36] The medium formula provided by the present invention can be adopted to remarkably decrease the contamination rate of *Acer pseudo-sieboldianum* explants, promote the growth of axillary buds, increase the proliferation coefficient and rooting rate. *Acer pseudo-sieboldianum* with excellent traits can be rapidly propagated by using the plant tissue culture method.

[37] The present invention improves the material and disinfection method of *Acer pseudo-sieboldianum* for the first time and reduces the contamination rate of inoculation.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[38] The present invention discloses a medium and method for tissue culture of *Acer pseudo-sieboldianum*, which may be achieved by those skilled in the art by reference to the content of this specification based on appropriate improvement of process parameters. In particular, all similar substitutions and modifications are obvious to those skilled in the art and are deemed to be included in the present invention. The method and application of the present invention have been described by preferred embodiments, and relevant personnel can obviously modify or appropriately change and combine the method and application described herein without departing from the content, spirit and

scope of the present invention to realize and apply the technology of the present invention.

[39] The medium raw materials or other reagents used in the medium and method for tissue culture of *Acer pseudo-sieboldianum* provided by the present invention are commercially available.

[40] The present invention will be further explained in detail below in combination with the example below:

[41] Example 1

[42] 1. Obtaining of explants

[43] On a sunny morning, the vigorous, pest-free branches of current year were taken, leaves were removed, and the branches were cut into 1.5 - 2cm stems with axillary buds as explants, then soaked in washing powder solution for 30 min and rinsed.

[44] 2. Sterilization

[45] The explants were treated with 5% sodium hypochlorite for 30 min, rinsed with sterile water, placed in sterilized culture flasks, sealed, placed in the refrigerator for 1 day and then taken out. The explants were treated with 70% alcohol on a super clean bench in a sterile room for 30 s, then disinfected with 0.1% HgCl2 for 30 min, and finally rinsed with sterile water 5 - 6 times.

[46] 3. Primary inoculation

[47] The explants were inoculated on the priming medium (basal medium + IBA 0.05 - 0.20 mg/L + sucrose 30 g/L). A temperature of a culture chamber was (24±1)℃, an illumination time is 12 h/d, and an illumination intensity was 2,000 Lx. During this period, observation was performed carefully, and contaminated materials were removed at any time to avoid cross-infection.

[48] The explants with axillary buds were inoculated in the MS medium or 1/2 MS medium supplemented with IBA of different concentrations (0 mg/L, 0.05 mg/L, 0.10 mg/L, 0.15 mg/L, and 0.20 mg/L). After inoculation, the growth conditions of axillary buds were observed and recorded every 5 days, and the following growth conditions were observed after 25 days.

[49] Table 1 Growth conditions of explants with axillary buds after inoculation

Treatment	1	2	3	4	5	6	7	8	9	10
Medium	MS	1/2MS	MS	1/2MS	MS	1/2MS	MS	1/2MS	MS	1/2MS
IBA (mg/L)	0	0	0.05	0.05	0.10	0.10	0.15	0.15	0.20	0.20
Germination rate (%)	10.2	11.3	39.5	42.2	68.7	90.2	67.6	66.8	58.3	58.9
Stem length (cm)	0.05	0.06	1.06	1.03	1.53	1.65	1.48	1.52	1.43	1.40

[50] The results show:

[51] Regardless of the MS or 1/2MS medium as a basal medium, when different concentrations of IBA are added, the germination rates of axillary buds and the stem

lengths increase first and then decrease with the increase of the IBA concentrations. When the IBA concentration is 0.10 mg/L, the germination rate of shoots and the elongation length of stems are higher than those in other treatments. In addition, the germination rate of shoots and the elongation length of stems in the 1/2 MS medium are slightly higher than those in the MS medium. Therefore, the 1/2MS medium supplemented with 0.10 mg/L IBA is the most suitable priming medium for culture of explants.

[52] 4. Proliferation culture

[53] The elongated axillary buds were cut and transferred to the proliferation medium (MS + IBA 0.05-0.2 mg/L+CPPU 0.5-1.5 mg/L + sucrose 30 g/L). The culture conditions remained unchanged.

[54] The proliferation medium was a MS medium, the concentrations of IBA were 0.05 mg/L, 0.10 mg/L and 0.20 mg/L, and the concentrations of NAA were 0.5 mg/L, 1.0 mg/L and 1.5 mg/L. After 20 days of culture, the statistical results of proliferation coefficient are as follows:

[55] Table 2 Proliferation of subculture for 20 days

Treatment	1	2	3	4	5	6	7	8	9
IBA (mg/L)	0.05	0.10	0.20	0.05	0.10	0.20	0.05	0.10	0.20
CPPU (mg/L)	0.5	0.5	0.5	1.0	1.0	1.0	1.5	1.5	1.5
Proliferation coefficient	1.36	1.95	1.89	3.15	4.06	2.96	2.51	2.96	2.68

[56] The results show that when the concentration of IBA is 0.1 mg/L and the concentration of CPPU is 1.0 mg/L, the proliferation coefficient reaches 4.06, which is significantly better than that in other treatments.

[57] 5. Rooting culture

[58] The cluster seedlings from the previous step were cut into individual seedlings and then inoculated into the rooting medium (basal medium + IBA 0.10 - 0.40 mg/L + sucrose 20 g/L) for rooting culture.

[59] For rooting culture, the MS medium or 1/2 MS medium was used and the concentrations of IBA were 0.10 mg/L, 0.20 mg/L, 0.30 mg/L and 0.40 mg/L. After 20 days of culture and observation, the following results are as follows:

[60] Table 3 Results of rooting culture test

Treatment	1	2	3	4	5	6	7	8
Medium type	MS	1/2MS	MS	1/2MS	MS	1/2MS	MS	1/2MS
IBA (mg/L)	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4
Rooting rate (%)	49.5	50.33	90.6	91.4	87.6	89.8	86.4	85.2

[61] The results show that when the medium is the 1/2 MS medium and the concentration of IBA is 0.2 mg/L, the plant root systems are robust and grow well, with a rooting rate of 91.4%, which is significantly better than that in other treatments.

WHAT IS CLAIMED IS:

1. A medium for tissue culture of *Acer pseudo-sieboldianum*, comprising a priming medium, a proliferation medium and a rooting medium, wherein

the priming medium is a 1/2MS medium containing 0.05 - 0.25 mg/L IBA and 25 - 35 g/L sucrose;

the proliferation medium is a MS medium containing 0.05 - 0.20 mg/L IBA, 0.5 - 1.5 mg/L CPPU and 25 - 35 g/L sucrose; and

the rooting medium is a 1/2MS medium containing 0.10 - 0.40mg/L IBA and 15 - 25g/L sucrose.

2. The medium of claim 1, wherein the priming medium is a 1/2MS medium containing 0.05 - 0.15 mg/L IBA and 28 - 32 g/L sucrose;

the proliferation medium is a MS medium containing 0.05 - 0.15 mg/L IBA, 0.8 - 1.2 mg/L CPPU and 28 - 32 g/L sucrose; and

the rooting medium is a 1/2MS medium containing 0.15 - 0.25 mg/L IBA and 18 - 22 g/L sucrose.

3. The medium of claim 1, wherein the priming medium is a 1/2MS medium containing 0.10 mg/L IBA and 30 g/L sucrose;

the proliferation medium is a MS medium containing 0.10 mg/L IBA, 1.0 mg/L CPPU and 30 g/L sucrose; and

the rooting medium is a 1/2MS medium containing 0.20 mg/L IBA and 20 g/L sucrose.

4. A method for tissue culture of *Acer pseudo-sieboldianum*, comprising the following steps:

taking and pretreating current-year branches of *Acer pseudo-sieboldianum* to obtain explants;

sterilizing the explants;

inoculating the sterilized explants into a priming medium for primary culture, wherein the priming medium is a 1/2MS medium containing 0.05 - 0.25 mg/L IBA and 25 - 35 g/L sucrose;

transferring axillary buds after primary culture to a proliferation medium for proliferation culture, wherein the proliferation medium is a MS medium containing 0.05 - 0.20 mg/L IBA, 0.5 - 1.5 mg/L CPPU and 25 - 35 g/L sucrose; and

transferring cluster seedlings after proliferation culture to a rooting medium for rooting culture, wherein the rooting medium is a 1/2MS medium containing 0.10 - 0.40 mg/L IBA and 15 - 25 g/L sucrose.

5. The tissue culture method of claim 4, wherein the pretreatment is as follows: the leaves of the current-year branches are removed, and the branches are divided into 1.5 - 2.0 cm stems with axillary buds, and washed.

6. The tissue culture method of claim 4, wherein the sterilization is as follows: the explants are treated with sodium hypochlorite for 20 - 40 min, then washed, sealed in sterilized culture flasks, and placed at 2 - 8°C for 20 - 30 h; after that, the explants are treated with alcohol for 20 - 40 s, then disinfected with mercuric chloride for 20 - 40 min, and finally rinsed with sterile water.

7. The tissue culture method of claim 6, wherein the sterilization is as follows: the explants are treated with sodium hypochlorite for 30 min, then washed, sealed in sterilized culture flasks, and placed at 4℃ for 24 h; after that, the explants are treated with alcohol for 30 s, then disinfected with mercuric chloride for 30 min, and finally rinsed with sterile water.

8. The tissue culture method of claim 6 or 7, wherein a mass volume percentage of the sodium hypochlorite is 3% - 7%; a volume percentage of the alcohol is 60% - 80%; and a mass volume percentage of the mercuric chloride is 0.08% - 0.12%.

9. The tissue culture method of claim 6 or 7, wherein the mass volume percentage of the sodium hypochlorite is 5%; the volume percentage of the alcohol is 70%; and the mass volume percentage of the mercuric chloride is 0.1%.

10. The tissue culture method of claim 4, wherein a temperature of the primary culture is 23 - 25℃, an illumination time is 12 h/d, and an illumination intensity is 2,000 Lx; a temperature of the proliferation culture is 23 - 25℃, an illumination time is 12 h/d, and an illumination intensity is 2,000 Lx; and a temperature of the rooting culture is 23 - 25℃, an illumination time is 12 h/d, and an illumination intensity is 2,000 Lx.

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ABSTRACT OF THE DISCLOSURE

The present invention relates to the technical field of tissue culture, in particular to a medium and method for tissue culture of *Acer pseudo-sieboldianum*. The medium includes a priming medium, a proliferation medium and a rooting medium, wherein the priming medium is a 1/2MS medium containing 0.05 - 0.25 mg/L IBA and 25 - 35 g/L sucrose; the proliferation medium is a MS medium containing 0.05 - 0.20 mg/L IBA, 0.5 - 1.5 mg/L CPPU and 25 - 35 g/L sucrose; and the rooting medium is a 1/2MS medium containing 0.10 - 0.40 mg/L IBA and 15 - 25 g/L sucrose. The medium formula provided by the present invention can be adopted to remarkably decrease the contamination rate of *Acer pseudo-sieboldianum* explants, promote the growth of axillary buds, increase the proliferation coefficient and rooting rate. *Acer pseudo-sieboldianum* with excellent traits can be rapidly propagated by the plant tissue culture method.