Optimization of *Limonium sinuatum* rooting by using multiplication media and pre-rooting media during tissue culture

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Abstract

Statice (*Limonium sinuatum*) is a popular ornamental flower crop around the world. Currently in HilverdaKooij, 4 different *L. sinuatum* cultivars (22016 Azur Wings; 8008 Blue Wings; 4004 Cobalt Wings; C024 tested pot plant) have a low rooting percentage (60-70%) in the micropropagation. This project is conducted to get a better understanding of the relationship among kind and level of multiplication media, pre-rooting media and rooting performance. By doing this project, HilverdaKooij wants to optimize rooting for these 4 different *L. sinuatum* cultivars. In this research, tissue culture stock clumps of these four cultivars were first multiplied on three different multiplication media. After 7 weeks, plants were cultured on three different pre-rooting media for 2 weeks, and then, plants were transferred to rooting medium for 3 weeks. The results showed medium 16-8 with NAA (1-napthaleneacetic acid) gave the best rooting percentage for the four cultivars. For cultivar 8008, the low concentration of BA (6-benzyladenine) used in multiplication stage improved root formation. For cultivar C024, NAA reduced the percentage of belt plants. Pre-rooting stage is recommended for cultivar 22016 and 8008 during micropropagation. For the future tissue culture of *L. sinuatum*, high concentration of NAA is recommended to use in the rooting stage, and cytokinins are omitted from rooting media. The outcome of this research can help HilverdaKooij solve the low rooting percentages problems of some *L. sinuatum* cultivars.

Key words: *Limonium sinuatum*, plant tissue culture, plant growth regulator, multiplication media, pre-rooting media, multiplication factors[1] (MF), rooting percentage

[1] Multiplication factors: the number of shoots that developed per shoot explant. Example, 5 shoot developed 10 shoots, the multiplication factors are 2.

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List of Abbreviations

BA – 6-benzyladenine

FeEDDHA – ethylenediamine di-2-hydroxy-phenyl acetate ferric

FeEDTA – ethylenediaminetetraacetate ferric sodium

MF – Multiplication factors

MM – Multiplication media

NAA– 1-napthaleneacetic acid

PM – Pre-rooting media

Chapter 1 Introduction

1.1 Background of HilverdaKooij

This research project is supported by HilverdaKooij which was created in 2008 following a merger between two renowned suppliers of plant material for cultivation of cut carnation. The cooperative drive behind HilverdaKooij has led to a powerful company supplying a unique assortment, with its main varieties being carnations, spray carnations, *Alstroemeria* and *Limonium*. Its large scale and network of branch offices in the Netherlands and abroad allow HilverdaKooij to provide its clients worldwide with a complete selection (HilverdaKooij, 2010a).

The laboratory in HilverdaKooij is focused on doing the tissue culture for all the commercial varieties of *Limonium* and new selections. The plant tissues are taken from the parents plants and initiated into clumps. Parts of the clumps are rooted at the head company in Netherlands. After rooting, the rooted shoots are delivered to the greenhouse for testing. Other parts of clumps are delivered to subsidiary (large scale tissue culture production labs) in other countries for multiplication and rooting (Fenyvesi, 2010). Current production labs are located in India (Pune) and China (Shanghai, Kunming).

1.2 General project background

Statice (*Limonium sinuatum*) has become a popular ornamental flower crop around the world in recent years. Conventional propagation of many cultivars is by side shoot or rooting cuttings, which take 6-8 months to develop with limited (20-30%) success (Fujita, 1993). So micropropagation has been developed to improve the propagating efficiency. Currently in HilverdaKooij, 4 different *L. sinuatum* cultivars (22016 Azur Wings; 8008 Blue Wings; 4004 Cobalt Wings; C024 Tested pot plant) have a low rooting percentage (60-70%) in the micropropagation. Also rooting ability is known to change with prolonged multiplication.

In tissue culture, the phase of multiplication and the rooting phase are usually opposite processes. In the first phase the plant has to make more plants, in the second phase the plant has to stop dividing and make roots. The transition between these phases can be difficult. There are plenty of researches about hormones' effect in tissue culture of different plants. However, most of them studied plant multiplication and rooting as two steps separately. There are only limited articles found that mention the phenomenon that multiplication hormones give an influence on the rooting stage. This project is conducted to get a better understanding of the

relationship between the multiplication media (MM) and rooting performance by using different concentration of growth regulators in MM. In this research, we also introduce a pre-rooting media (PM). PM is an intermediate between multiplication and rooting. It aims to makes a better transition from multiplication stage to rooting stage. In this research different combinations of multiplication and pre-rooting hormones will be tested, and the impact from multiplication step to rooting process will be analyzed.

1.3 Research Objectives

This project aims to optimize rooting for these 4 different *L. sinuatum* cultivars by using the different concentration of multiplication media and pre-rooting media.

1.4 Research Questions

1. What effects do the different multiplication media have on each *L. sinuatum* cultivar?

- What are the multiplication factors of each *L. sinuatum* cultivar on the different multiplication media?
- What is the morphology of each *L. sinuatum* cultivar on the different multiplication media?

2. What effects do the 3 different pre-rooting media have on rooting of the 4 different *L. sinuatum* cultivars?

- What is the morphology of 4 different *L. sinuatum* cultivars on the 3 different pre-rooting media?
- What is the rooting rate of 4 different *L. sinuatum* cultivars after being placed on the 3 different pre-rooting media?

3. Which are the most optimal combinations of multiplication, pre-rooting and rooting media for the rooting of 4 different *L. sinuatum* cultivars?

4. Is there a relationship between multiplication parameters and rooting efficiency?

Chapter 2 Literature review

2.1 Background of *Limonium sinuatum*

The genus *Limonium*, formerly called statice, is a member of the family Plumbaginaceae, which consists of 150 wild species. Wild species of statice are widely distributed in the coastal areas and plains throughout the world in both tropical and temperate zones such as Europe, the Middle East, Latin America, Africa, China and Japan (Bailey, 1978). The tiny flowers are narrow and funnel shaped, consisting of loose terminal panicles appearing in loose bows. The leaves of the *L. sinuatum* plants form a rosette pattern at the base of the plant; the leaves grow close to the ground. Prior to flowering the stem elongates and terminal leaves become upright. Stems are winged and grow approximately 70-80 cm tall when flowering (HilverdaKooij, 2010b). Table 2.1 shows the growing habit of the four tested *L. sinuatum* cultivars in greenhouse. Every year on average HilverdaKooij sells 1 million *L. sinuatum* young plants to the growers worldwide. The main market is in the Netherlands, Japan, Colombia and Israel (Strooper, 2010).

Table 2.1 Four tested *L. sinuatum* cultivars' growing habits in greenhouse (HilverdaKooij, 2010c)

	22016	**8008**	**4004**	**C024**
Speed of growth	Normal[2]	Normal	Normal	Normal
Height of crop	Long[3]	Medium[4]	Long	Short[5]
Flower size	Medium[6]	Medium	Medium	Medium
Productivity	Very High[7]	Very High	High[8]	High

[2] Normal speed of growth: it takes 4 months from young plants to the plants have flowers.
[3] Long length crop: 75cm
[4] Medium length crop: 70cm
[5] Short length crop: 30cm
[6] Medium flower size: 5cm
[7] Very high production: per plant produces more than 30 stems per year
[8] High production: per plant produces 20-30 stems per year

2.2 Plant tissue culture technique and its application

2.2.1 Background of plant tissue culture

The definition of the plant tissue culture is: culture of plant tissues excised from the parent body on a nutrient medium under sterile conditions; the medium contains usually inorganic and organic nutrients, plant growth regulators and vitamins (de Klerk, 2009).

Generally a complete plant tissue culture process involves the following 5 stages:

1. Mother plant selection and preparation
2. Initiation- establishing an aseptic culture
3. Multiplication- the production of suitable propagules
4. Rooting- preparation for growth in the natural environment
5. Transfer to the *ex vitro* environment (George *et al*., 2008)

2.2.2 Current application of tissue culture technique in horticulture production

In the past 50 years, tissue culture has become a major tool in agriculture. Currently, the major uses of commercial tissue culture are:

1) Mass propagation of specific clones, especially those developed by breeders and geneticists who want to rush new plants into the marketplace.
2) Preparation of parent stock plants that produce desired hybrid seeds.
3) Maintaining disease-free germ plasm.
4) Keeping plant in year round production by bypassing their natural seasonal cycles (Ingels, 2001).

By tissue and organ culturing, many ornamental plants have found their way to the marketplace. A survey of European plant tissue culture laboratories in 1996 and 1997 already lists nearly two thousand plant genera, species or cultivars grown in 312 official and 193 commercial laboratories (Laimer *et al*., 2003).

2.3 Multiplication hormones and rooting hormones

2.3.1 Plant growth regulators

Some chemicals occurring naturally within plant tissues have a regulatory, rather than a nutritional role in growth and development. These compounds, which are

generally active at very low concentrations, are known as *plant hormones*. They control the shape of the plant and mediate growth response on external factors such as light and temperature. Synthetic chemicals with similar physiological activities to plant growth substances, or compounds having an ability to modify plant growth by some other means are usually termed *plant growth regulators*. There are several recognized classes of plant growth regulators. Until relatively recently only five groups were recognized namely auxins, cytokinins, gibberellins, ethylene and abscisic acid (George *et al.,* 2008).

The growth, differentiation and organogenesis of plant tissue become feasible only on the addition of one or more of these classes of hormones to a medium. The ratio of hormones required for root or shoot induction varies considerably with the tissue (Razdan, 2003). Auxins and cytokinins are by far the most important for regulating growth and morphogenesis in plant tissue and organ cultures.

2.3.2 Auxin

Auxin is very widely used in plant tissue culture. It is required by most plant cells for cell division, cell elongation and root initiation. At the cellular level, auxin controls basic processes such as cell division and cell elongation. Since they are capable of initiating cell division they are involved in the formation of meristems (Barz *et al.*, 1977).

The most commonly used auxins are IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), 2,4-D (2,4-dichlorophenoxyacetic acid) and NAA (1-napthaleneacetic acid) (George *et al*., 2008).

Though belonging to the same class, they are usually used for different purposes. Together with cytokinins, 2,4-D is used primarily for callus induction. IAA, NAA and IBA are usually used for root induction without cytokinin (Davies, 1995).

2.3.3 Cytokinins

Cytokinins cause cell division. Such cell division can lead to shoot regeneration *in vitro,* by stimulating the formation of shoot apical meristems and shoot bud. Cytokinins also play a role in prevention of senescence and reversion of the deteriorating effect of auxins on shoots. Generally, a high concentration of cytokinin will block root development (Blakesley *et al*., 1987).

The commonly used cytokinins are BA (6-benzyladenine), Kinetin, 2-ip (2-isopentyladenine), ZT (zeatin) and TDZ (thidiazuron) (Skoog *et al*., 1980). Although used in research, the natural cytokinins 2-ip and ZT are not used by

commercial laboratories routinely, because of their cost. Accordingly, they are not tested in this research.

2.3.4 Auxin-Cytokinin interaction

Many aspects of cellular differentiation and organogenesis in tissue and organ cultures have been found to be controlled by an interaction between auxin and cytokinin concentrations. The balance between the two kinds of regulators is most often required for the formation of adventitious shoots and root meristems (Negrutiu *et al*., 1978). This is illustrated in Fig. 2.1.

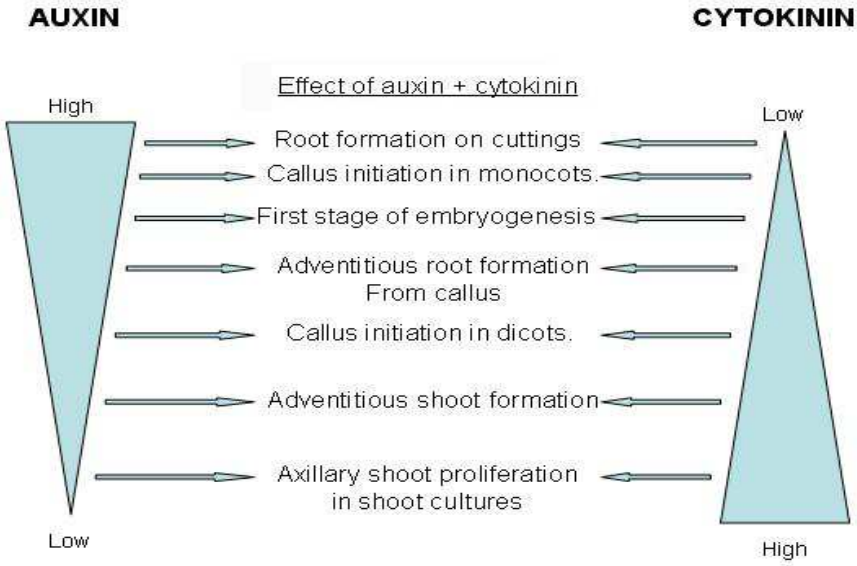


Figure. 2.1 The relative concentrations of auxin and cytokinin typically required for growth and morphogenesis in plant tissue culture (George *et al*., 2008).

Concluded from the above literature, the requisite concentration of each type of regulant differs greatly according to the plant species, the cultural conditions and the compounds used; the interactions between the two classes of regulators are often complex, and more than one combination of substances can produce optimum results (Skoog *et al*., 1957).

In a rooting medium, the kind and concentration of growth regulators (mostly auxins) are designed to make the balance suitable for rooting formation. But the hormones which accumulated in the plant tissue during the previous multiplication stage may shift the growth regulator balance. This is a basic understanding of how the multiplication hormone used influences rooting.

Chapter 3 Materials and Methods

3.1 Plant Materials

Four *L. sinuatum* cultivars (22016 Azur Wings; 8008 Blue Wings; 4004 Cobalt Wings; C024) were used in this research. They were originally initiated from inflorescence stems from the greenhouse, and then routinely cultured *in vitro* on Murashige and Skoog (MS) medium for more than 6 months. Tissue culture of these cultivars is micro-propagated every year in the laboratory. Part of these stocks clumps will be provided for this experiment.

3.2 Media

Table 3.1 The different multiplication media, pre-rooting media and rooting media used in this research.

Cultivar	Multiplication media (7 weeks)	Pre-rooting media (2 weeks)	Rooting media (3 weeks)
22016	17-7, 17-8, 17-9[a]	16-6, 16-7, 16-8 20-8 control medium	20-8
8008	17-7, 17-8, 17-9	16-6, 16-7, 16-8 20-8 control medium	20-8
4004	17-7, 17-8, 17-9	16-6, 16-7, 16-8[b]	20-8
C024	18-0, 18-5, 19-4	16-6, 16-7, 16-8 20-8 control medium	20-8

a. The media names lack specificity due to confidentiality of the work. The numbers refer to the medium preparation sheet.

b. There were not enough clumps available so 4004 without control treatment.

Multiplication media:
Multiplication media group for cultivar 22016, 8008 and 4004
-17-7-: low BA medium
-17-8-: medium BA medium
-17-9-: high BA medium

Multiplication media group for cultivar C024[9]
-18-0-: high BA medium (with iron source FeEDTA[10])
-18-5-: low BA medium (with iron source FeEDTA)
-19-4-: same as -18-0- (with iron source FeEDDHA)

3 different pre-rooting media:
-16-8-: NAA medium
-16-6-: low kinetin medium
-16-7-: high kinetin medium

1 control medium:
-20-8- NAA medium, same as rooting medium

1 rooting medium:
-20-8- NAA medium

3.3 Methods

3.3.1 Desk research

To conduct this project, I searched the data on HilverdaKooij, *L. sinuatum* and tissue culture from books, handouts and internet. I also documented these references during the desk research.

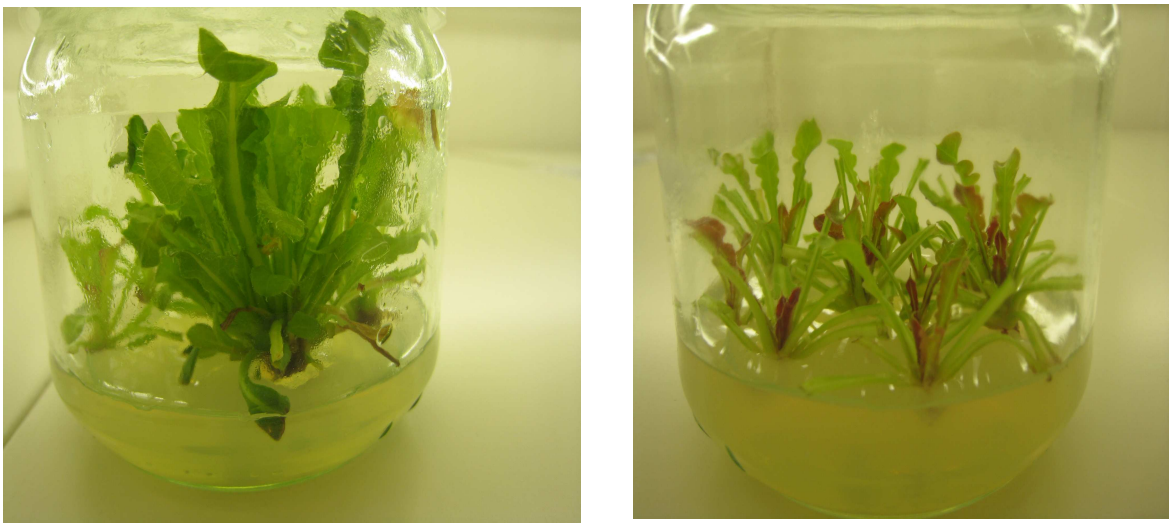
3.3.2 Research in Laboratory

1) **Multiplication:** Clumps were separated into single plants. Long leaves were cut short by using the knife (see Figure 3.1). Each culture vessel (6 x 12cm glass bottles containing 70ml of medium) was considered as one replication, and seven plants were cultured per vessel. There were 3 replicates per treatment. Each cultivar was multiplied on a set of 3 different MM. After being cultured on same media for 2 cycles (3.5 weeks per cycle), the plants were put on PM. During the multiplication stage, the morphology of each cultivar and abnormal phenomena of plants, like belt plants[11] and vitrification (leads to plant death) were observed. The multiplication factor (MF)[12] was calculated.

[9] C024 is a pot type plant, and therefore it has a different response *in vitro*. This multiplication media group is especially designed for pot type *L. sinuatum*. The concentrations of growth regulators in this group of media are lower than media group for cultivar 22016, 8008 and 4004.
[10] HilverdaKooij already wanted to know a lot of things and where short of clumps. Therefore, tested FeEDDHA only on the fastest most sensible cultivar where its effect was expected most likely to be noticeable
[11] Meristems grow in the straight line
[12] Multiplication factors: the number of shoots that developed per shoot explant. Example, 5 shoot developed 10 shoots, the multiplication factors are 2.

2) **Pre-rooting:** The multiplied stock was placed on the three different pm and control medium. Clumps were separated into single plants. The long leaves were cut short. Single plants were placed in culture vessel, and 7 single plants per vessel. There were 10 replications per treatment. After two weeks the plants were transferred to rooting media. During the pre-rooting phase, observation of the morphology, the abnormal phenomena of each cultivar, MF and the number of explants forming roots was recorded.

3) **Rooting:** Single plants were placed in culture vessel without shorting the leaves, and 10 single plants per vessel. There were 3 replicates per treatment. The time for rooting was 3 weeks. During rooting, the number of rooted plants was counted every 2 or 3 days.



Before cutting *After cutting*

Figure 3.1 The multiplication and pre-rooting stages showing the morphology of *L. sinuatum* before and after cutting *in vitro* (Zhao Hang, 2010)
(* The newly formed shoots at the end of cycle are smaller than the main shoots.)

3.3.3 Data analysis

Experiment was set up in a completely randomized design. Data analysis was done using Statistical Product and Service Solutions 17.0 (T-Test, ANOVA Test, and Post Hoc Test). The significant difference was at 5% level.

Chapter 4 Results

4.1 Cultivar 22016

4.1.1 Multiplication stage of cultivar 22016

The study on MF of cultivar 22016 on three different MM is presented in Table 4.1. It showed that plants on MM 17-8 and 17-9 had significantly higher MF than plants on medium 17-7 (P=0.010). For the percentage of vitrified plants there were no significant differences between three MM (P=0.063), although medium 17-9 had 4.15% vitrified plants. No belt plants occurred on the three MM.

Table 4.1 *L. sinuatum* cultivar 22016's multiplication factors, percentage of vitrified plants and belt plants on three different multiplication media during multiplication stage*

Multiplication media	Multiplication factors	Percentage of vitrified plants	Percentage of belt plants
17-7	1.46 b	0.0% a	0.0%
17-8	1.99 a	0.0% a	0.0%
17-9	1.89 a	4.15% a	0.0%

* Data represent mean values of three replications per treatment. Mean values followed by the same subscript letters indicate treatments are not significant different at P ≤ 0.05.

4.1.2 Pre-rooting stage of cultivar 22016

The MF of plants on pm 16-7 and 16-8 were significantly higher than medium 16-6 (P=0.048) (Table 4.2) (Figure 4.1).

Table 4.2 *L. sinuatum* cultivar 22016's multiplication factors on three pre-rooting media during pre-rooting stage*

Pre-rooting media	Multiplication factors
16-6	1.26 b
16-7	1.69 a
16-8	1.55 a

* Data represent mean values of three replications per treatment. Mean values followed by the same subscript letters indicate treatments are not significant different at P ≤ 0.05.

PM 16-6 PM 16-7 PM 16-8

Figure 4.1 The morphology of *L. sinuatum* cultivar 22016 on three different pre-rooting medium in multiplication stage (Zhao Hang, 2010)

4.1.3 Rooting stage of cultivar 22016

During rooting stage, plants from MM 17-9 with PM 16-8 had a significantly higher rooting percentage (77.7%) than plants directly from medium 17-9 (27.3%) (P=0.003). Plants directly from MM 17-9 (control) also started rooting one week later than plants with PM 16-8. The interaction between MM and PM was not significant at 5% level on the basis of ANOVA (P=0.263). The rooting percentage was only affected by three different PM (P=0.000). Plants on PM 16-8 had the highest rooting percentage of 68.4% (Table 4.3). PM 16-7 gave the lowest rooting percentage (13.1%) among the three PM. For this cultivar, plants started rooting between 12 and 17 days after being transferred to rooting media (Figure 4.2).

Table 4.3 *L. sinuatum* cultivar 22016's rooting percentage on the three pre-rooting media during rooting stage*

Media	Rooting percentage
17-8 (control treatment)	30.0% c
17-9 (control treatment)	27.3% c
16-6	43.0% b
16-7	13.1% d
16-8	68.4% a

* Data represent mean values of three replications per treatment. Mean values followed by the same subscript letters indicate treatments are not significant different at $P \leq 0.05$.

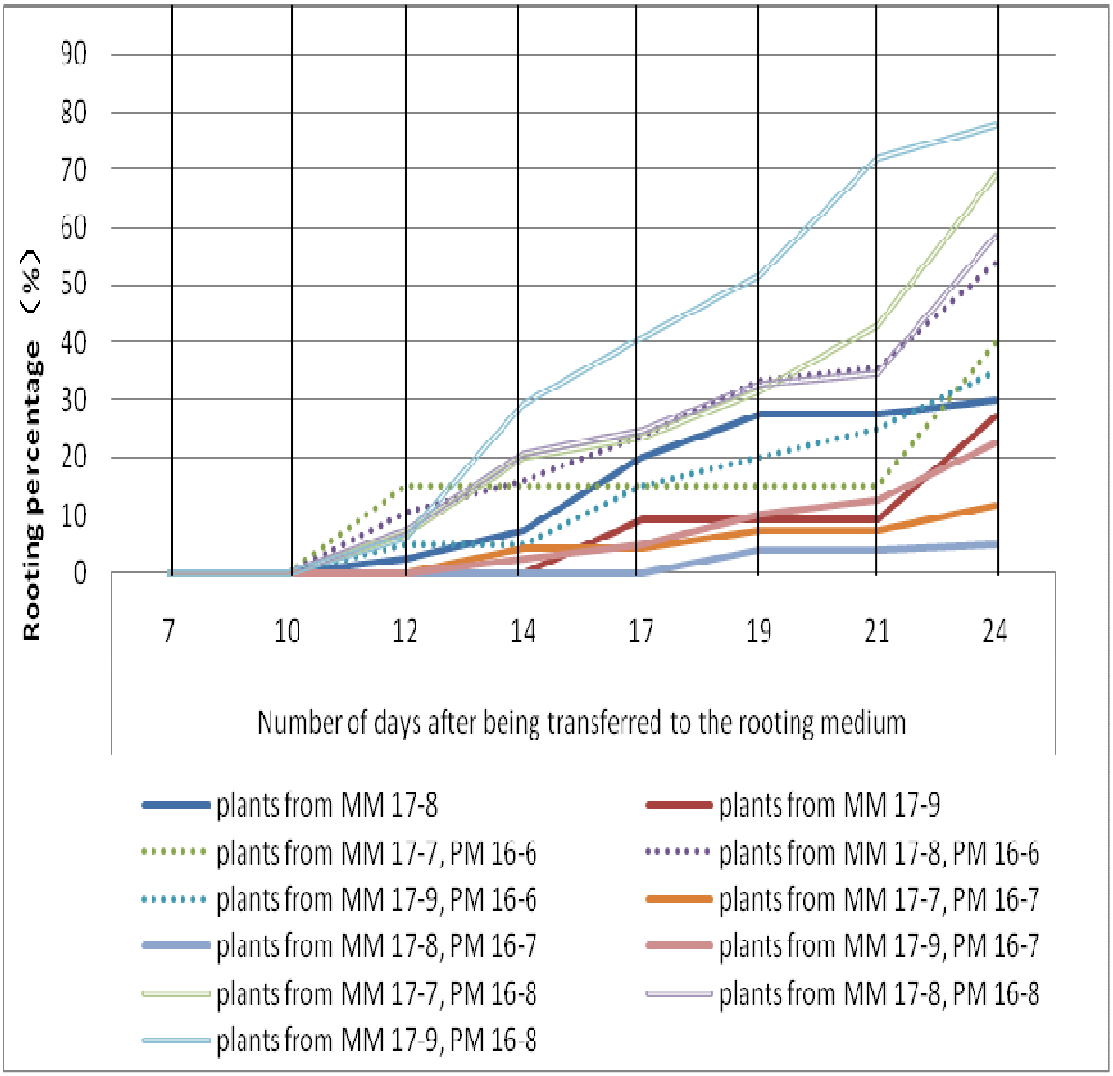


Figure 4.2 The rooting percentage of cultivar 22016 from different multiplication media and pre-rooting media during rooting stage

4.2 Cultivar 8008

4.2.1 Multiplication stage of cultivar 8008

As with previous cultivar 22016, MM 17-8 and 17-9 had no significant difference in MF (P=0.422). They were significantly higher than plants on medium 17-7 (P=0.010). There was no significant difference in percentage of vitrified plants between three MM (P=0.063), although medium 17-9 had 2.1% vitrified plants. No belt plants occurred on the three MM (Table 4.4) (Figure 4.3).

Table 4.4 *L. sinuatum* cultivar 8008's multiplication factors, percentage of vitrified plants and belt plants on three different multiplication media during multiplication stage*

Multiplication media	Multiplication factors	Percentage of vitrified plants	Percentage of belt plants
17-7	1.47 b	0.0% a	0.0%
17-8	2.16 a	0.0% a	0.0%
17-9	1.84 a	2.1% a	0.0%

* Data represent mean values of three replications per treatment. Mean values followed by the same subscript letters indicate treatments are not significant different at $P \leq 0.05$.

MM 17-7 MM 17-8 MM 17-9

Figure 4.3 The morphology of *L. sinuatum* cultivar 8008 on three different multiplication media in multiplication stage (Zhao Hang, 2010)

4.2.2 Pre-rooting stage cultivar 8008

The three different PM did not give the significant difference in MF (P=0.138) (Table 4.5).

Table 4.5 *L. sinuatum* cultivar 8008's multiplication factors on three pre-rooting media during pre-rooting stage*

Pre-rooting media	Multiplication factors
16-6	1.41 a
16-7	1.20 a
16-8	1.36 a

* Data represent mean values of three replications per treatment. Mean values followed by the same subscript letters indicate treatments are not significant different at P ≤ 0.05.

4.2.3 Rooting stage of cultivar 8008

There was an interaction between MM and PM (P=0.001). Plants from medium 17-7 with PM 16-8 had the significantly highest rooting percentage (47.3%) compared to all the treatments. After being transferred to rooting medium, it had the fastest response among all treatments. Rooting started after 7 days (Figure 4.4).

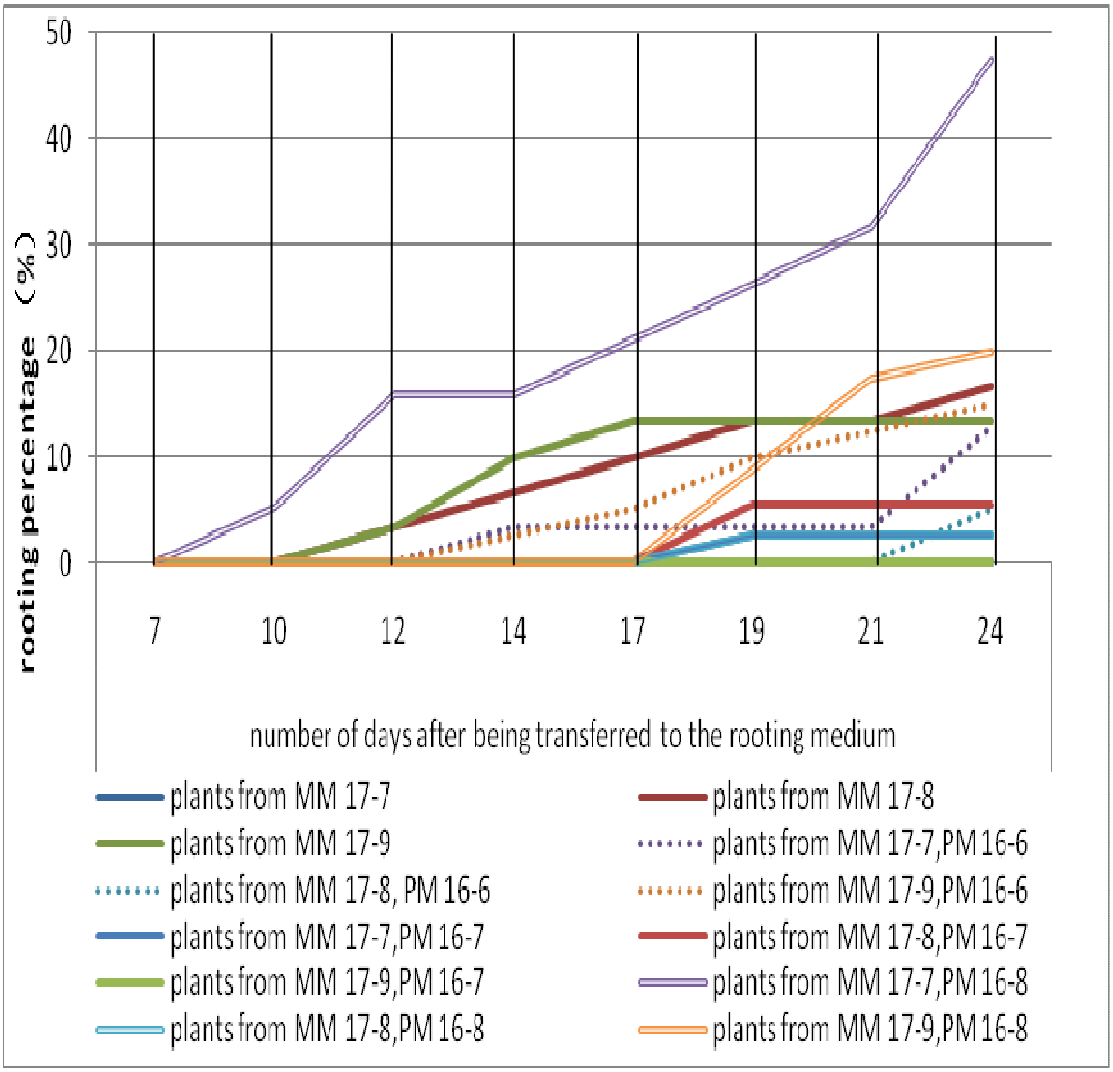


Figure 4.4 The rooting percentage of cultivar 8008 from different multiplication media and pre-rooting media at rooting stage

4.3 Cultivar 4004

4.3.1 Multiplication stage of cultivar 4004

There was no significant difference in MF between three MM (P=0.943). No vitrified plants and belt plants occurred on the three MM (Table 4.6) (Figure 4.5).

Table 4.6 *L. sinuatum* cultivar 4004's multiplication factors, percentage of vitrified plants and belt plants on three different multiplication media during multiplication stage*

Multiplication media	Multiplication factors	Percentage of vitrified plants	Percentage of belt plants
17-7	1.35 a	0.0%	0.0%
17-8	1.48 a	0.0%	0.0%
17-9	1.53 a	0.0%	0.0%

* Data represent mean values of three replications per treatment. Mean values followed by the same subscript letters indicate treatments are not significant different at $P \leq 0.05$.

MM 17-7 MM 17-8 MM 17-9

Figure 4.5 The morphology of *L. sinuatum* cultivar 4004 on three different multiplication media in multiplication stage (Zhao Hang, 2010)

4.3.2 Pre-rooting stage of cultivar 4004

There was no significant difference in MF between three PM (P=0.158) (Table 4.7).

Table 4.7 *L. sinuatum* cultivar 4004's multiplication factors on three pre-rooting media during pre-rooting stage*

Pre-rooting media	Multiplication factors
16-6	1.08 a
16-7	1.08 a
16-8	1.05 a

* Data represent mean values of three replications per treatment. Mean values followed by the same subscript letters indicate treatments are not significant different at P ≤ 0.05.

4.3.3 Rooting stage of cultivar 4004

The interaction between MM and PM was not significant at 5% level (P=0.159). However, the rooting percentage was significantly affected by the three different PM (P=0.000). Medium 16-8 gave the highest rooting percentage of 89.5% (Table 4.8). Medium 16-7 had the lowest rooting percentage (8.70%) among three media. For this cultivar, plants with PM 16-8 started rooting 14 days after being transferred to rooting medium (Figure 4.6).

Table 4.8 *L. sinuatum* cultivar 4004's rooting percentage on the three pre-rooting media during rooting stage*

Pre-rooting media	Rooting percentage
16-6	55.8% c
16-7	8.70% b
16-8	89.5% a

* Data represent mean values of three replications per treatment. Mean values followed by the same subscript letters indicate treatments are not significant different at P ≤ 0.05.

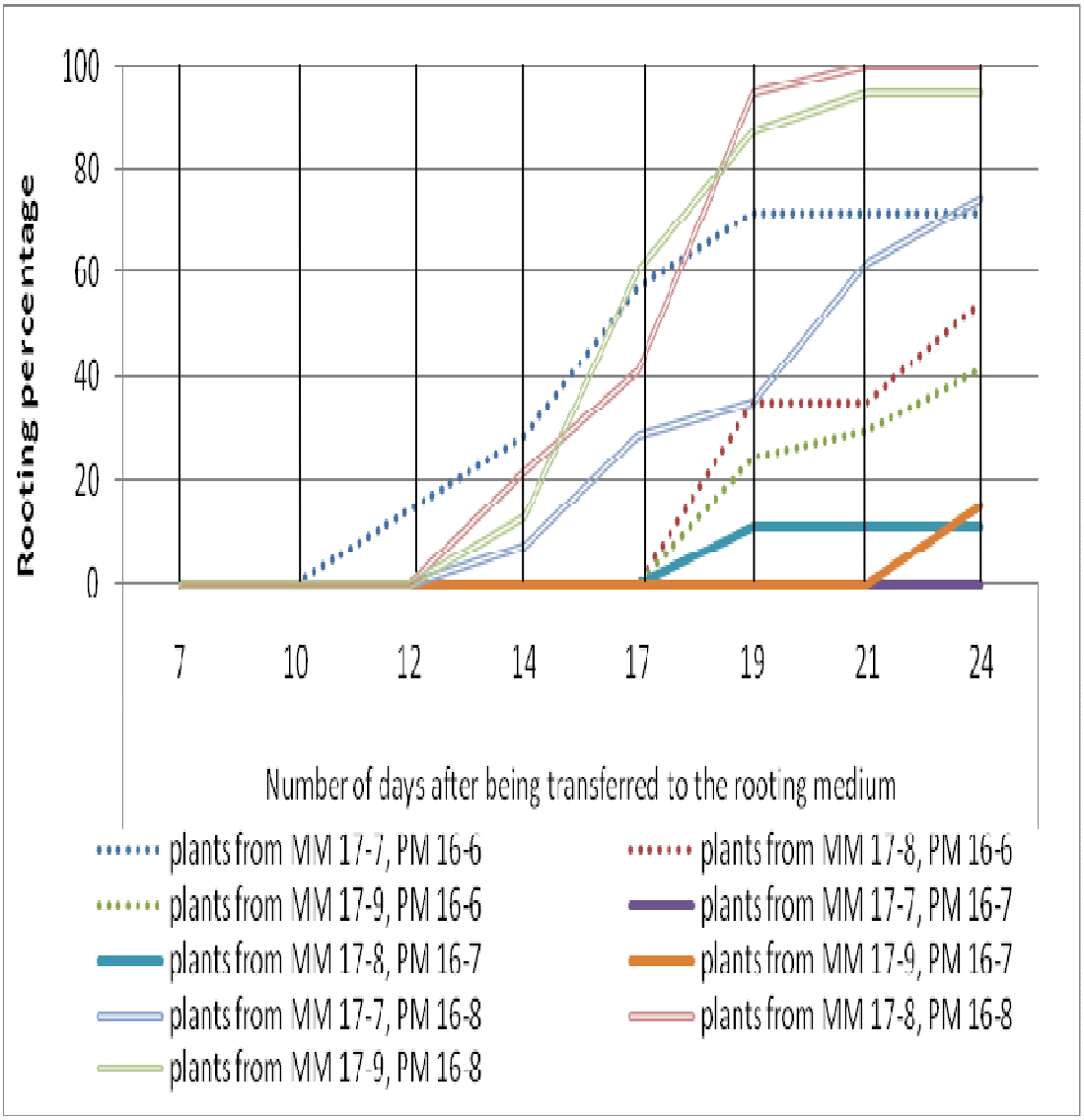


Figure 4.6 The rooting percentage of cultivar 4004 from different multiplication media and pre-rooting media at rooting stage

4.4 Cultivar C024

4.4.1 Multiplication stage of cultivar C024

The MF of plants on medium 18-0 were significantly higher than plants on media 18-5 and 19-4 (P=0.040). There was no significant difference in percentage of vitrified plants and belt plants between the three MM (P=0.197 and P=0.685 respectively) (Table 4.9), but compared with previous cultivars, this cultivar produced obviously more vitrified and belt plants.

Table 4.9 *L. sinuatum* cultivar C024's multiplication factors, percentage of vitrified plants and belt plants on three different multiplication media during multiplication stage*

Multiplication media	Multiplication factors	Percentage of vitrified plants	Percentage of belt plants
18-0	2.45 a	0.0% a	17.4% a
18-5	1.87 b	5.5% a	13.8% a
19-4	2.04 b	3.7% a	10.3% a

* Data represent mean values of three replications per treatment. Mean values followed by the same subscript letters indicate treatments are not significant different at P ≤ 0.05.

4.4.2 Pre-rooting stage of cultivar C024

The three PM did not give the significant difference in MF (P=0.117). The three PM had no influence on the percentage of vitrified plants (P=0.197). For the belt plants, they were affected by different PM (P=0.001). Significantly less belt plants occurred on the PM 16-8 (Table 4.10) (Figure 4.7).

Table 4.10 Cultivar C024's multiplication factors, percentage of vitrified plants and belt plants on three different pre-rooting media during pre-rooting stage*

Pre-rooting media	Multiplication factors	Percentage of vitrified plants	Percentage of belt plants
16-6	2.33 a	3.3% a	11.3% a
16-7	2.04 a	5.7% a	17.3% a
16-8	1.81 a	0.0% a	3.2% b

* Data represent mean values of three replications per treatment. Mean values followed by the same subscript letters indicate treatments are not significant different at P ≤ 0.05.

PM 16-6 PM 16-7 PM 16-8

Figure 4.7 The morphology in pre-rooting stage of *L. sinuatum* cultivar C024 on three different pre-rooting media (Zhao Hang, 2010)

4.4.3 Rooting stage of cultivar C024

All the treatments started rooting between 7 and 10 days after being transferred to rooting medium. There was no interaction between MM and PM (P = 0.915). Rooting percentage was significantly affected by PM (P = 0.006). Plants on PM 16-8 had the highest rooting percentage of 98.9% (Table 4.11). Medium 16-7 gave the lowest rooting percentage (81.7%) among three PM (Figure 4.8).

Table 4.11 Cultivar C024's rooting percentage on the three pre-rooting media during rooting stage*

Pre-rooting media	Rooting percentage
16-6	92.5% b
16-7	81.7% c
16-8	98.9% a

* Data represent mean values of three replications per treatment. Mean values followed by the same subscript letters indicate treatments are not significant different at P ≤ 0.05.

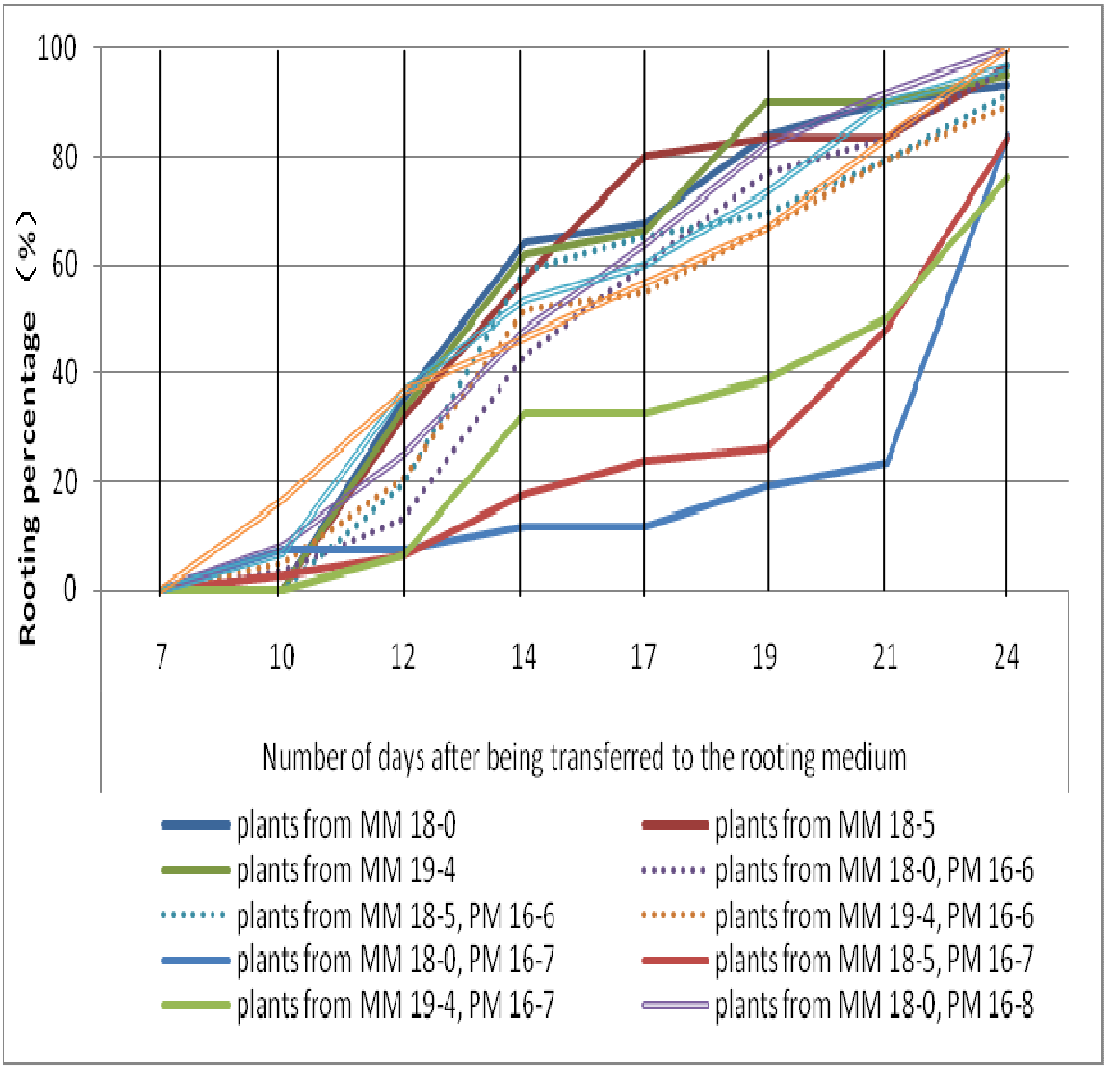


Figure 4.8 The rooting percentage of cultivar C024 from different multiplication media and pre-rooting media at rooting stage

Chapter 5 Discussion

5.1 Cultivar 22016

High levels of BA induce shoots (Slater *et al*., 2003). Media 17-8 and 17-9 contained higher concentration of BA, therefore, they had higher MF than plants on medium 17-7. Table 4.1 showed that vitrified plants were not significantly affected by different levels of BA. But vitrified plants both occurred on high level of BA for cultivar 22016 and 8008. Maybe high level of BA had effect on vitrification. George (2008) had reviewed that cytokinin promote cell division. High concentrations of cytokinin cause the level of some species to induce shoots to become hyperhydric.

In the pre-rooting stage, low concentration of Kinetin slows the rate of plants to induce new shoots more than NAA does (Table 4.2). No article has mentioned this phenomenon so far. Generally, the effect of cytokinins on tissue culture can vary according to the level of cytokinins used (Razdan, 2003).

In the rooting stage, Figure 4.2 showed the medium with NAA induced roots and speeded up root formation. The high level of Kinetin blocked the roots induction. In general, auxins induce roots, and cytokinins at high level inhibit root formation (de Klerk, 2009).

5.2 Cultivar 8008

In the multiplication stage, cultivar 8008 had a similar result as cultivar 22016 (Table 4.4).

In the pre-rooting stage, Kinetin was ineffective in promotion of shoot formation (Tablele 4.5). Razdan, (2003) reported that cytokinins are a complex class of plant hormones. Kinetin has less effect on shoot induction than BA does.

In the rooting stage, low concentration of BA used in the multiplication stage and NAA used at pre-rooting stage is a considerable combination for rooting. This combination speeded up root formation (Figure 4.4). Auxin promotes root induction (Davies, 1995). A balance between auxin and cytokinin growth regulators is most often required for the formation of root (Hansen *et al*., 1985) (Figure 2.1).

5.3 Cultivar 4004

Cytokinins did not have an obvious effect on the MF during multiplication stage and pre-rooting stage (Table 4.6 and 4.7). The effect of cytokinins on tissue culture can vary according to the variety of plant (George *et al*. 2008). The vitrification and belt plants appeared to not be the problem for this cultivar (Table 4.6).

In the rooting stage, high level of Kinetin used in pre-rooting media had a negative effect on root formation. The medium supplemented with NAA could improve root formation (Figure 4.6). At high concentration of cytokinin inhibits or delays root formation (Blakesley *et al*., 1987). Auxins at high levels induce roots (Slater *et al*., 2003). There were not enough plants tested for this cultivar. In the future, the control treatment will be introduced as a comparison. The result of this research did not prove whether it is necessary to introduce a pre-rooting stage for the rooting of cultivar 4004.

5.4 Cultivar C024

In the multiplication stage, at the same concentration of BA, media with FeNAEDTA iron source had higher MF than media with FeEDDHA (Table 4.9). During multiplication, FeEDDHA could reduce chlorosis, increase content of chlorophyll and iron but have no effect on the number of side shoots (Kim *et al*., 2003). Cultivar C024 is a difficult pot type plant. It is not easy to cut clumps into single plant. The way of cutting could also have an influence on the result.

Seen from Table 4.10 cultivar C024 was sensitive to Kinetin. High concentration of Kinetin causes high percentage of belt plants, and NAA could reduce the percentage of belt plants. Plants that multiplied too much developed the belt plants. Cytokinins promote cell division, and high concentrations of cytokinin cause the level of some species to have an unusual shape. Auxin reduces shoot formation and improves root formation (de Klerk, 2009).

In the rooting stage, plants cultured on media with NAA or without NAA started rooting at same time and gave the similar rooting percentage (Figure 4.8). Maybe the reason was cultivar C024 became an easy rooting cultivar after the multiplication media already had been adapted from the standard multiplication media. This cultivar easily became vitrified and produced belt plants. However, it started rooting fastest and had the highest rooting percentage among the four cultivars.

Chapter 6 Conclusion and Recommendations

For cultivar 22016, plant multiplication and rooting are two separate stages. For multiplication stage, multiplication media 17-8 and 17-9 are the best. Pre-rooting medium 16-8 is recommended as an intermediate between multiplication and rooting.

For cultivar 8008 the optimized combination for rooting is multiplication media 17-7 used at multiplication stage and pre-rooting medium 16-8 used at pre-rooting stage is recommended. For the future production, it would be better to use multiplication media 17-8 or 17-9 for multiplying, and use multiplication medium 17-7 before culturing plants on pre-rooting medium 16-8. After conducting this research cultivar 8008 still has the low rooting percentage problem. Therefore, more researches still need to be conducted to improve rooting percentage.

For cultivar 4004, multiplication stage and rooting stage seems to have no relationship. For multiplication there are no best multiplication media found to produce high MF. But multiplication media 17-8 or 17-9 are recommended for multiplying. For rooting the best pre-rooting medium is 16-8.

For cultivar C024, there is no relationship between multiplication phase and rooting phase. Multiplication medium 18-0 is recommended during multiplication stage. And it is unnecessary to introduce pre-rooting media during tissue culture. This will also be an advantage to save production time.

For the future research, in order to make the result more reliable, it is better to have more than 4 replicates per treatment. For the future tissue culture of *L. sinuatum*, high concentration of NAA is recommended to use in the rooting stage, and cytokinins are omitted from rooting media.

This research shows the phenomena that multiplication hormone gives the influence on rooting stage. This can contribute to further tissue culture experiments about *L. sinuatum* rooting and other tissue culture crops. The outcome of this research can help HilverdaKooij solve the low rooting percentages problems of some *L. sinuatum* cultivars, and this can contribute to a more efficient production system for the company.

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