Effects of Explants Division by Cutting, Concentrations of TDZ and Number of Sub-culture Cycles on Propagation of 'Kluai Hom Thong' Banana in a Temporary Immersion Bioreactor System

Sarisa Daungban, Paweena Pumisutapon and Nopmanee Topoonyanont*

Faculty of Science, Maejo University, Nongharn, Sansai, Chiang Mai 50290

Poonpat Poonnoy

Faculty of Engineering and Agro-Industry, Maejo University, Nongharn, Sansai, Chiang Mai 50290

Abstract

Banana is an industrial fruit crop for which there is strong demand in export markets. Because of disease problems in growing areas, there is a need for more pathogen-free planting materials. To develop a system for propagating bananas in a temporary immersion bioreactor (TIB) we studied factors affecting the number of new shoots formed by *Musa* (AAA group) 'Kluai Hom Thong' in a 700-ml twin-flasks TIB with liquid medium supplied for 2 minutes a time every 4 hours, in comparison to the same types of explants cultured in a conventional semi-solid medium system. We compared leaving the explants uncut or longitudinally cutting them into 2 or 4 sections and tested adding thidiazuron (TDZ) at the concentrations of 0, 0.125 and 0.250 mg l^{-1}), for 3 culture cycles of 6 weeks each. The results showed that the most effective combination was cutting the explants in 4 sections and culturing in TIB with 0.125 mg l^{-1} TDZ, which resulted in up to 11.03 new shoots per explant in the third culture cycle. The number of shoots per container increased to 48 and 132 in the second and third cycles, respectively, or 2.08 and 5.74 times more than the number of shoots per container in the first cycle (23). Bananas cultivated in the semi-solid medium system developed fewer new shoots per container and a larger percentage of small-sized shoots.

Keywords: micropropagation; apical dominance; thidiazuron; subculturing

1. Introduction

'Kluai Hom Thong' banana (*Musa* AAA group) is an important industrial fruit crop in Thailand. There is very strong demand for bananas in the export market. At present, Thailand's largest export market for bananas is Japan. It is estimated that there is demand for 8,000 tons of bananas a year in the Japan

*Corresponding author: nopmanee@mju.ac.th

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market, but Thailand can export only 1,000 tons a year (Customs Department, 2013). Bananas are susceptible to diseases such as *Cucumber mosaic virus* and Panama disease, which can spread quickly and cause great damage to plantations (Wongkrut, 2011). Farmers thus need sources of disease-resistant varieties or pathogen-free planting materials. Wanjan (2010) reported that when rhizomes from 3-month-old banana suckers were cut into 2, 3, or 4 sections, new sprouts would arise in 3 weeks, making it possible to increase the number of banana plants more quickly.

Tissue culture is a practical technique for producing large quantities of pathogen-free planting materials. Most of the researches on tissue culture of banana have focused on the use of different types of cytokinins to induce shoots. For instance, Shirani *et al*. (2009) reported that the most effective cytokinin concentrations for micropropagating commercial cultivars of banana were 2 μM TDZ and 22.2 μM BAP. While higher concentrations of TDZ ($\geq$ 5 μM) and BAP ($\geq$ 33.3 μM) induced greater numbers of shoots, but a large proportion of the shoots were morphologically abnormal.

Nevertheless, conventional system using semi-solid medium has rather high costs, especially labor cost (Topoonyanont *et al*., 1995) and is too slow to keep up with the demand for industrial-scale production. The temporary immersion bioreactor (TIB) system (Alvard *et al*., 1993) is a faster alternative for producing large volumes of new plants in the laboratory. The TIB system is superior to conventional tissue culture mainly, because it helps reduce labor costs, which account for 60-70 % of total cost. TIB systems are now widely used for the propagation of several economic crops, such as potato (Jiménez *et al*., 1999), sugarcane (Topoonyanont *et al*., 2012) and pineapple (Firoozabady and Gutterson, 2003). As for the use of a TIB system for propagating banana, Roels *et al*. (2005) experimented on different lengths of time for supplying medium (4, 12 or 22 minutes), different frequencies of supplying medium (every 3, 5 or 7 hours) and the effect of different plant growth regulators (PGRs) - benzyladenine (BA), meta-topolin (MET) and TDZ. They found that MET at the concentration of 44.4 μM was the most effective in inducing shoots, but the multiplication rate of new shoots was slow after 6 subcultures when the plants were subcultured every 28 days. The frequency and duration of supplying medium had no effect on the propagation rate. Shoot height, number of leaves, and number of roots were not significantly different from banana plants propagated using a conventional system.

The objective of this research was to develop an efficient system for micropropagating 'Kluai Hom Thong' banana, comparing the use of a twin-flasks TIB with a conventional system, as well as comparing different concentrations of TDZ and methods of dividing the initial explants, with the aim of maximizing the number of usable shoots.

2. Materials and methods

The starting material for explant sources

consisted of 6-week-old shoots of *Musa* (AAA group) 'Kluai Hom Thong' (4-10 cm in height) cultured on modified semi-solid MS medium (Murashige and Skoog, 1962) with 1 mg l^{-1} BA, 3 % sucrose and 3 g l^{-1} gellan gum, adjusted to pH 5.8. The cultures were maintained at 25±2 ° C with light from fluorescent light bulbs (40 µmol $m^{-2}s^{-1}$) for 14 hours per day.

The experiment was designed as factorials in CRD. The factors were methods of cutting the shoot (no cutting, cut into 2 sections and cut into 4 sections), concentrations of TDZ (0, 0.125 and 0.250 mg l^{-1}), and culture methods (TIB or semi-solid medium), for a total of 3*3*2 = 18 treatment combinations, with 4 replications per treatment combination.

For uncut shoots, the leaves were cut off, leaving about 1.5 cm of shoot. For the cut shoots, the leaves were also cut off; leaving about 1.5 cm of shoot, and then the shoot was cut longitudinally through the bud to divide it into 2 or 4 equal sections. For both cut and uncut shoots, total of 4 shoots were put in each container, so the vessels contained either 4 uncut shoots or 8 or 16 cut shoot sections each.

For the TIB system, the explants were cultured in 700 ml capacity twin glass bottles filled with 300 ml medium. The culture medium was the same as mentioned above but without gellan gum. In the TIB system, liquid medium was supplied every 4 hours for 2 minutes a time. The TIB system was compared to a conventional system.

The experiment lasted 18 weeks, with 3 culture cycles of 6 weeks each. After the first 6-week period, only new shoots that measured 4-10 cm in height (Figure 1A) were harvested and used as the explants for the subsequent cycle. They were either left uncut or were cut into 2 or 4 sections and cultured in the same treatment combination as before for another 6 weeks. However, for the third cycle the 0.250 mg l^{-1} TDZ treatment was discontinued because during the second cycle a large percentage of micro shoots (Figure 1C) developed in the semi-solid medium at this concentration of TDZ, so there were not enough large shoots to continue the experiment as before.

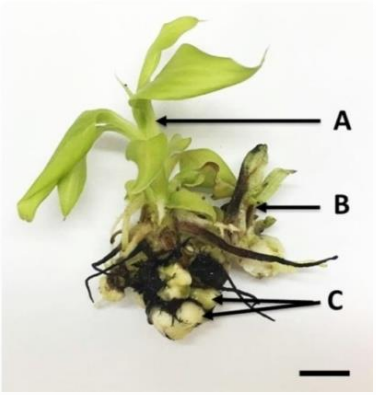

Figure 1 Large shoot (A), small shoot (B) and micro shoots (C) arising from 'Kluai Hom Thong' banana explants in the in vitro multiplication stage (bar = 1 cm)

For each culture cycle, data were recorded on the number of new shoots per explant, number of shoots per container, and percentage of each kind of shoots (large, small or micro). The micro shoots were transferred to hormone-

free modified MS medium in a TIB system and cultured for 6 weeks to stimulate shoot elongation.

For statistical analysis of the data, ANOVA was performed using SPSS program. Means were compared in one-way analysis to find statistical significance at 95 % confidence level.

3. Results

3.1 Types of shoots

In both the TIB and semi-solid medium systems, 3 types of shoots were observed: Large shoot, small shoot and micro shoot. Large shoots were 4-10 cm tall with clearly expanded leaves and fully formed roots (Figure 1A). Small shoots were 2-4 cm tall, with curled leaves, no roots, and usually occurred in clumps (Figure 1B). Micro shoots were no more than 1 cm tall, appearing as small protuberances (Figure 1C). Only the large shoots were suitably large to use as starting materials for the subsequent steps of the experiment.

3.2 Survival rate of explants

In all 3 culture cycles, for both the TIB system and the semi-solid medium system, the survival rate of uncut shoots was the highest at 100 %, because the shoots were not damaged by cutting. For the shoots that were divided into 2 or 4 sections, the survival rate was less than 100 % but still over 80 %, because the cutting process entailed some damage to the shoots, which caused some of them to die (data not shown).

3.3 Percentage of different types of shoots

The percentage of different types of shoots in the different treatments differed during each culture cycle. From Table 1 it was shown that for explants that were not cut, the new shoots that arose in all 3 culture cycles were 100% large shoots, in both TIB and semi-solid medium, and regardless of the concentration of TDZ. When the explants were divided by cutting into 2 or 4 pieces, small shoots and micro shoots also developed during the second and third culture cycles, except no micro shoots were observed in the treatments without TDZ in the medium, for both TIB and semi-solid culture.

For the semi-solid medium system, in medium with no TDZ added, no small or micro sized shoots arose during the second culture cycle, but in the third cycle a large proportion of small shoots was observed. In the second cycle a large percentage of micro shoots arose in the treatments with 0.25 mg l^{-1} TDZ (54.49 and 68.15% for shoots cut in 2 and 4 pieces, respectively), so there were not enough large shoots to sub culture for the third cycle. Even at the lower concentration of 0.125 mg l^{-1} TDZ, the semi-solid medium system treatments resulted in a large percentage of micro shoots in the third culture cycle (45.20 and 63.18 % for shoots cut in 2 and 4 parts, respectively).

3.4 Shoots per explant

The results showed that dividing the shoot by cutting resulted in more new shoots, and the number of new shoots tended to increase with increasing concentration of TDZ. From Figure 2, it was observed that regardless

of whether they were cultured in medium with TDZ or not, the uncut explants gave rise to only 1-1.5 shoots per explants, while the explants that were cut into 2 and especially 4 pieces sprouted more new shoots with increased concentration of TDZ. The explants cultured in TIB with 0.125 mg l^{-1} TDZ gave rise to an average of 3 and 3.77 new shoots in the second cycle and 11.03 and 9.5 new shoots in the third cycle for explants that were divided in 2 pieces and 4 pieces, respectively.

Table 1 Percentage of large shoots (L), small shoots (S) and micro shoots (M) arising from 'Kluai Hom Thong' banana explants cultured in a TIB system or on semi-solid modified MS medium with different concentrations of TDZ through 3 culture cycles, each lasting 6 weeks

TDZ (mg l^{-1})	Shoot Cutting type	Cycle 1			Cycle 2			Cycle 3		
		L	S	M	L	S	M	L	S	M
TIB system										
0	No cut	100.00	0	0	100.00^{a}	0.00^{j}	0.00^{i}	100.00^{a}	0.00^{i}	0.00^{e}
	2-section cut	100.00	0	0	67.28^{c}	32.72^{e}	0.00^{i}	51.22^{c}	49.78^{c}	0.00^{e}
	4- section cut	100.00	0	0	72.33^{c}	27.77^{d}	0.00^{i}	45.79^{d}	55.31^{b}	0.00^{e}
0.125	No cut	100.00	0	0	100.00^{a}	0.00^{j}	0.00^{i}	100.00^{a}	0.00^{i}	0.00^{e}
	2- section cut	100.00	0	0	58.60^{bc}	38.32^{b}	4.18^{h}	51.39^{c}	40.21^{e}	9.30^{d}
	4- section cut	100.00	0	0	46.61^{bc}	47.29^{a}	7.10^{g}	41.64^{f}	44.13^{d}	15.23^{c}
0.250	No cut	100.00	0	0	100.00^{a}	0.00^{j}	0.00^{i}	NA	NA	NA
	2- section cut	100.00	0	0	41.35^{c}	31.12^{f}	28.53^{f}	NA	NA	NA
	4- section cut	100.00	0	0	13.24^{c}	34.55^{d}	53.31^{c}	NA	NA	NA
Semi-solid medium system										
0	No cut	100.00	0	0	100.00^{a}	0.00^{j}	0.00^{i}	100.00^{a}	0.00^{i}	0.00^{e}
	2- section cut	100.00	0	0	100.00^{a}	0.00^{j}	0.00^{i}	62.46^{b}	38.54^{f}	0.00^{e}
	4- section cut	100.00	0	0	100.00^{a}	0.00^{j}	0.00^{i}	43.65^{e}	57.35^{a}	0.00^{e}
0.125	No cut	100.00	0	0	100.00^{a}	0.00^{j}	0.00^{i}	100.00^{a}	0.00^{i}	0.00^{e}
	2- section cut	100.00	0	0	34.26^{b}	28.15^{d}	38.69^{e}	22.44^{g}	23.46^{c}	45.20^{b}
	4- section cut	100.00	0	0	27.43^{bc}	27.43^{g}	46.14^{d}	17.36^{h}	20.52^{b}	63.18^{a}
0.250	No cut	100.00	0	0	100.00^{a}	0.00^{j}	0.00^{i}	NA	NA	NA
	2- section cut	100.00	0	0	27.15^{c}	19.36^{i}	54.49^{b}	NA	NA	NA
	4- section cut	100.00	0	0	8.52^{c}	24.33^{h}	68.15^{a}	NA	NA	NA
		NS								

Within a colum means followed by the same letter(s) are not significantly different at 5% level by Duncan multiple rang test.

The results from the semi-solid medium system followed the same trend as those from the TIB system, but the number of shoots per explants was generally less, i.e. 1-1.45 for uncut explants. In the second cycle, the highest number of shoots per explant in the semi-solid medium system was 1.56 for explants cut in 2 pieces cultured with TDZ at 0.125 mg l^{-1} and 5.60 shoots for explants cut in 4 pieces and cultured with TDZ at 0.125 mg l^{-1}. In the third cycle, for explants cut in 2 and 4 pieces and cultured with TDZ at 0.125 mg l^{-1} the mean number of shoots per explant was 1.25 and 7.13, respectively. For the treatments with TDZ at the rate of 0.250 mg l^{-1}, the total number of shoots per explant was high but the proportion of micro shoots was too high, so the 0.25 mg l^{-1} TDZ treatments were discontinued in the third cycle of the experiment (Figure 2).

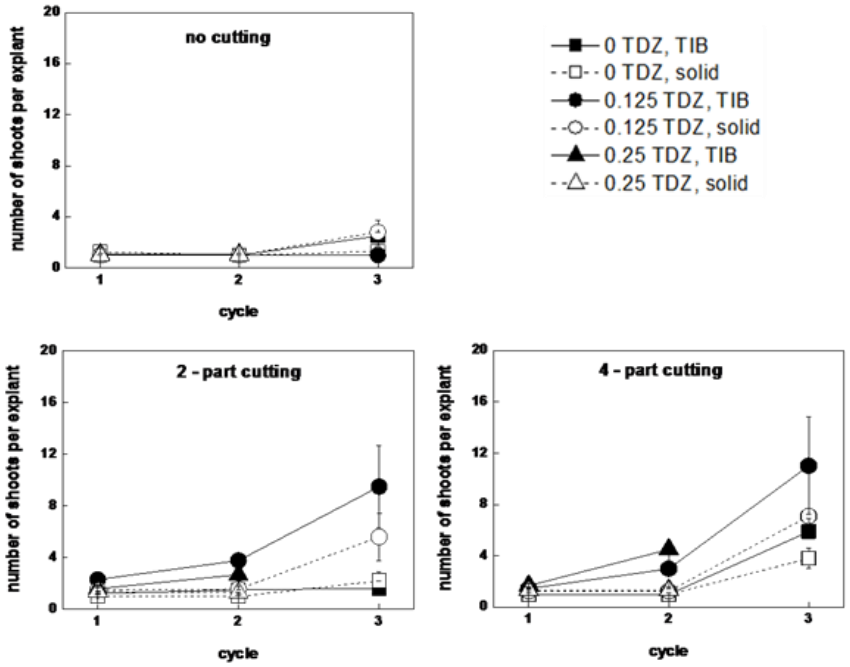

Figure 2 Number of shoots per explant of 'Kluai Hom Thong' banana shoots (uncut, cut into 2 or 4 sections) cultured in semi-solid modified MS medium or liquid medium in TIB with different concentrations of TDZ added for 3 culture cycles of 6 weeks duration each.

3.5 Number of shoots per container

In Figure 3 it was shown that when uncut shoots were cultured in the TIB system with both concentrations of TDZ, very few new shoots were formed (4-6.2 shoots per container). Shoots that were divided by cutting grew more new shoots. In particular, when the explants were cut into 4 parts and cultured in medium with 0.125 mg l^{-1} TDZ, the number of shoots per container increased with each culture cycle,

from 23 in Cycle 1 to 48 and 132 in Cycle 2 and Cycle 3, respectively, or an increase of 2.08 and 5.74 times.

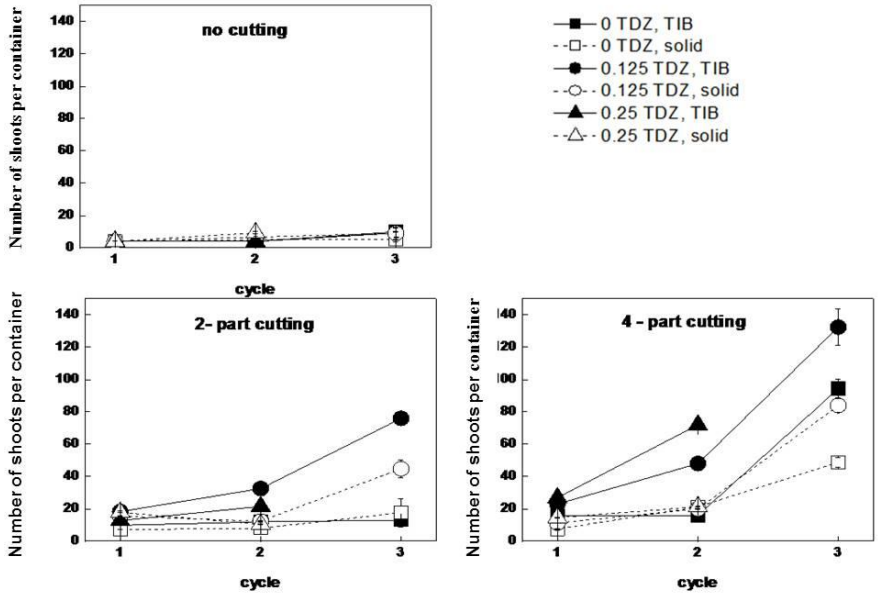

Figure 3 Number of shoots per container of 'Kluai Hom Thong' banana shoots (uncut, cut into 2 or 4 sections) cultured in semi-solid modified MS medium or liquid medium in TIB with different concentrations of TDZ added for 3 culture cycles of 6 weeks duration each.

3.6 Elongation of micro shoots

Micro shoots were very small plants with unfurled leaves. We observed their growth by culturing the micro shoots in a TIB system with PGR-free medium. After 6 weeks the micro shoots could grow into large shoots with expanded leaves and long, full roots (Figure 4). When transferred to the greenhouse, these new shoots were successfully acclimatized and grew normally (data not shown).

4. Discussion

In this study we found that the factors of dividing the explant by cutting and adding different concentrations of TDZ to the medium had a significant effect on the number of new banana plants that could be propagated in the TIB system. Cutting each bud into 4 pieces can make propagation much more efficient. The results are consistent with report of Ngomuo *et al.* (2014) found that the banana split topped only by varying the diameter of the top four can increase the number of shoots the most. While no cut explants clearly show less number of shoots. This is probably because the apical bud is damaged by the cutting, so apical dominance is released and thus lateral buds can fully develop (Barker and Steward, 1962).

There were reports in *Hippeastrum* sp. cultured *in vitro* that number of new shoots increased when apical bud of shoot explant was longitudinally cut (Ephrath *et al*., 2001; Zhu *et al*., 2005). In addition, explant-cutting will also increase the surface area contact between the parts of the plant to medium. It may absorb nutrients better (Ngomuo *et al*., 2014). Although some of the cut shoots died from the damage, the survival rate was greater than 80 % and the number of new shoots per container could be easily doubled or quadrupled during the first culture cycle simply through cutting.

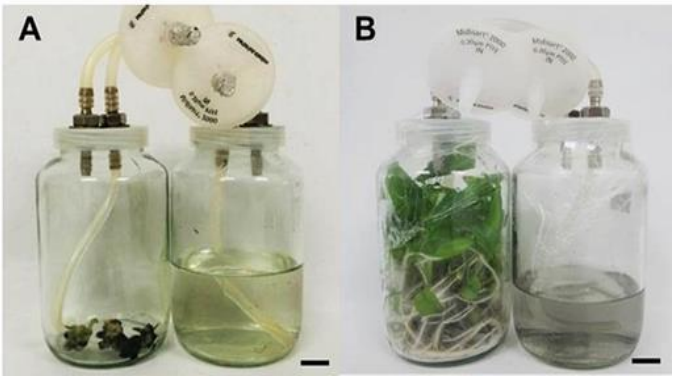

Figure 4 Excised micro shoots from the experiment (A) elongated to form large shoots (B) after being cultured with PGR-free modified MS medium in a TIB system for 6 weeks (bar = 1 cm).

The comparison between TIB and semi-solid medium system is great different in this study. Using liquid medium under TIB conditions results in a significantly higher multiplication rate compared to semi-solid medium. This is consistent with research that found in several of the TIBs can increase the propagation rate of banana over the using semi-solid medium (Roels *et al*., 2005) and other plants, such as the pineapple (Escalona *et al*., 1999), *Eucalyptus* (McAlister *et al*., 2005), vanilla (Ramos-Castella *et al*., 2014) and hybrid chestnut (Vidal *et al*., 2015).

However, in the multiplication stage, it is necessary to be aware of the residual effects of PGRs. In previous study, Roels *et al*. (2005) reported that there was cumulative number of shoots and buds formed after each subculture in plantain (*Musa* AAB) using MET on cytokinin stimulation increases the amount found to be likely caused many small amounts consistently clear, especially after a 4th round until round 10 in TIB and semi-solid medium. In this research we used TDZ, which is classed as a phenylurea cytokinin (Phenyl-3-(1,2,3-thiadiazol-5-ylurea)). TDZ works by inhibiting cytokinin oxidase (Jones and Schreiber, 1997), the enzyme that normally breaks down endo-genous cytokinins in plants. When cytokinin oxidase is inhibited, the level of endogenous cytokinins accumulates. When TDZ is added to the medium repeatedly, the level of cytokinin may get too high, resulting in multiple shoot formation. From our results it was observed that in the first culture cycle, when TDZ was added to the medium, initially the number of

new banana shoots that formed was not very high, but when the new large shoots were divided a second time for the second culture cycle, the number of new shoots rose greatly. At the concentration of 0.250 mg l^{-1} TDZ especially, there were many new shoots, including micro shoots. At the concentration of 0.125 mg l^{-1} TDZ the number of new shoots was perhaps more appropriate.

It is interesting whether micro shoots of banana arises from shoot cutting or TDZ can elongate to normal morphology, they were useful because they could elongate and form fully functional leaves and roots when they were sub-cultured in PGR-free medium. Shirani *et al*. (2009) found that high concentrations of cytokinin (>33.3 μM TDZ and >5 μM BA), caused higher abnormality in banana culture. Howawer in our experiments we used TDZ at much lower concentrations (0.125-0.25 mg l^{-1} = 0.56-1.145 μM) which abnormality of bananas was not observed. It is reported by Ventachalam *et al*. (2007) that no bananas have an abnormality morphology characteristics and genetic variation from the cytokinin handling highly concentrated (BA or kinetin up to 10 mg l^{-1}).

5. Conclusion

Our study demonstrates the appropriate micropropagation method of banana to support industrial production systems cultivar from TIB. We recommend the following protocol for propagating 'Kluai Hom Thong' banana in TIB system. Start by removing the leaves from banana shoots and cutting them longitudinally through the bud into 4 sections. Place 16 explants per bottle in semi-solid MS medium with 0.250 mg l^{-1} TDZ (300 ml per container) and culture for 6 weeks. Separate the new shoots, cut each large shoot in 4 pieces again, and place them in the TIB system with liquid MS medium with only 0.125 mg l^{-1} TDZ (300 ml per container). This way you can obtain a maximum of 132 new shoots per container. It is possible to continue sub-culturing in this manner indefinitely. When you want to transfer banana plants to the greenhouse, simply transfer the shoots to liquid MS medium with no PGRs added in the TIB system for 6-8 weeks, and they will develop into large shoots with strong root systems that are ready for acclimatization.

6. Acknowledgements

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