Chapter 5

Discussions

Part I Studies on factors influencing growth and *in vitro* tuberization.

1. Shoot growth

Leaf emergence and expansion

After transferring the initial cultured seedlings onto various tested media and/or placing in different illuminating conditions, the seedlings developed chlorophyll pigments in their young leaves except in the seedlings placing in the darkness which were still white in color, known as etiolated seedlings. This physiological change normally occurs when proplastids or etioplasts, containing prolamellar bodies that carry the precursor pigment for chlorophyll, have been exposed to light. They are further developed to chloroplasts via the stimulation of chlorophyll synthesis by a cytokinin, soon after exposure to light and development of thylakoids and grana from the prolamellar bodies (Wikipedia, 2007d). Further development, the emergence of young leaves and their expansion was related to the osmotic potential of a culture medium that greatly influenced by sugar adding. The high concentration of sucrose adding, at 7 %, gave high osmotic potential over the optimal level for leaf growth and development. Similarly, an addition of coconut water at the highest level tested at 20 % resulted in delaying leaf emergence for 2 weeks. However, an addition of coconut water gave a slight effect, when compared to that from the direct sucrose adding, since it has been reported that coconut water comprises various sugars, e.g. fructose, glucose and sucrose at 5.25, 7.25 and 9.18 g/l, respectively, also 8 g/l mannitol and 15 g/l sorbitol, the concentrations varies with maturity and/or cultivars (Arditti and Ernst, 1993). Thus, coconut water at 20 % gave less change in the osmotic potential of the culture medium. An increase in the medium osmotic potential, undoubtedly has resulted in decreasing water uptake into the plant tissues, and then further affects growth and development, and also organ formation (Pierik, 1977; Arditti and Ernst, 1993). Similarly, Ket *et al*. (2004) reported that growth rate of the jewel orchid (*Anoectochilus formosanus*) decreased with more negative water potential relating with sucrose concentration in the culture medium, where the growth and development generally increased with increasing sugar concentration until an optimum and then decreased at very high concentration.

Addition of some plant growth regulators, IAA at 0.01 – 1.00 mg/l, had not significantly affected the emergence and expansion of young leaves. Similar to the results obtained from adding low concentrations of the cytokinin, BAP at 0.01 – 0.05 mg/l. However, increasing BAP concentrations from 0.1 to 2.0 mg/l had relatively more effects in delaying leaf emerging and its expansion. But with coconut water, it seems to reduce the BAP effects. The results from these experiments showed that

high concentrations of BAP at 0.1 – 2.0 mg/l, decreasing in auxin : cytokinin ratios strongly stimulated cell division of meristematic tissue of *in vitro* shoot apex, and further proliferated to form protocorm-like bodies (Figure 5 and 6). These physiological changes helped to suppress growth of the shoot apex and leaf primordia. While adding coconut water, a rich source of auxins and cytokinins, and other substances of cytokinin-like activities supported the changing in the balance of auxin and cytokinin, and then suppressed the effect of BAP. It is widely known that the ratio of auxin to cytokinin in plant tissues determines their differentiation and development, such as initiation of shoot versus root buds (Wikipedia, 2007a)

Shoot height and leaf size

The sucrose concentrations had not affected shoot height and number of leaf, but the highest concentration tested at 7 % affected leaf size, in that the increased high osmotic potential caused by the supra-optimal sucrose concentration suppressed water uptake and turgidity during cell growth and enlargement, which later showed an adverse effect on the unfavorable leaf size. However, the seedlings could still be grown on the medium without sucrose, because they got some sugar from the coconut water which was a basal ingredient of the medium in the experiment 1. While in the experiment 5, the seedlings cultured on the medium without coconut water could still have some sugar to support their growth and development by hydrolysis of starchy reservoirs in their protocorm tissues which had been accumulated since they germinated on the sowing medium, or derived from *in vitro* photosynthesis in the functional leaf exposing to light. The seedlings cultured in the absence of sucrose produced big leaves reasoning an increase in photosynthetic leaf area, similar to those obtained from seedlings of *Habenaria rhodocheila* cultured in a medium without sucrose produced big leaves, while sucrose at the highest concentration tested, i.e. 8 % affected a decrease in leaf size (Piyatrakul, 2004). Contrarily, protocorms of *Paphiopedilum concolor* could not develop into seedlings when cultured in a medium without sucrose and coconut water supplements (Phornsawatchai, 1992).

Coconut water at the tested levels in Experiment 2 did not significantly promote shoot height, number of leaf and also leaf size but showed a slight trend to improve leaf growth. However, adding coconut water at 15 % in Experiment 3 showed a significant effect on shoot growth in terms of shoot height, leaf width and leaf length, because coconut water is a liquid endosperm that contains various complex substances, e.g. vitamins, amino acids and growth regulators. It has been reported extensively to add into *in vitro* culture media of various orchid cultures to promote their growth (Arditti and Ernst, 1993; Pierik, 1977). Similarly, Nualkaew (2004) reported that adding coconut water at 15 – 30 % promoted shoot growth of *Brachycorythis* sp., while in the absence coconut water plantlets showed a decrease in shoot height and leaf size. The same result was found in *Paphiopedilum concolor* cultured in the media supplemented with 10 – 20 % coconut water showing an increase in seedling canopy and leaf width (Phornsawatchai, 1992).

Addition of auxin, IAA at 0.01 - 1.0 mg/l had not significantly influenced plantlet growth, similar to the result caused by BAP at low concentrations at 0.01 - 0.10 mg/l. Contrarily, increasing BAP to higher concentrations supported increases in shoot height, but decreased in leaf size; nevertheless, when having coconut water, the

BAP effectiveness was retarded. Because high BAP concentrations promotes cell division, and/or inhibits cell elongation (Rappaport, 1978), the coconut water incorporated to the culture medium changed the balance of auxin and cytokinin to favour cell growth. According to Nualkaew (2004), seedlings of *Brachycorythis* sp. showed no shoot growth when cultured in a medium supplemented with NAA at 0.5, 1.0 and 2.0 mg/l, or with BAP at 0.5 – 1.0 mg/l alone or both in combinations, the seedlings died when supplemented with higher BAP concentrations, i.e. 2.0 – 4.0 mg/l both in the absence or presence of NAA at 0.5 – 2.0 mg/l.

Regarding illuminating effects, the plantlets cultured in continuous darkness gave more plant height and leaf length than culturing in light, but had not effected on number of leaf. These basic physiological changes occurred similar to seed germination in the dark, in that it caused cell elongation at the elongation zone of hypocotyl and young leaf. This is the results of cooperative actions of gibberellins and auxins, abundantly produces in the meristematic tissues of seedling young shoot and young leaf obtained from the dark. The actions increase cell wall elasticity and water uptake into the cell and further results to increase cell size, especially in its length (Rappaport, 1978; Roberts and Hooley, 1988). In addition, plantlets exposing to the light decreases in the production of gibberellins (Wikipedia, 2007c), and then reduces in shoot height and leaf length.

2. Tuberization and growth

The supplement of sucrose at the highest concentration, i.e. 7 % affected time of *in vitro* tuberization, and also a decreased in tuberization percentage. Similarly, the plantlets cultured in the medium without sucrose and coconut water under a dark condition, slowly produced tuber and tuberization percentage was decreased. Because the high sucrose concentration gave supra optimal level and unfavorable osmotic potential condition for cell water uptake, and then affected tuber formation of the seedlings. While under the poor conditions, no supplement with any kind of carbon source and lighting, the plantlets continued to use their starchy reservoirs, which was insufficient to support their growth and development, due to photosynthesis could not take place in the dark to produce sucrose and some chemical energy, resulting in decreases in their growth and tuberization. Sugar is very important component and essential for *in vitro* growth and development (Pierik, 1977; Rasmussen, 1995), it can be utilized in the synthesis of other essential compounds or used as sources of energy and respiration (Arditti, 1992). According to *in vitro* cultured potato, sucrose played a dual role to support a suitable carbon source and also provided a favorable osmotic condition for tuber growth and development (Khuri and Moorby, 1995).

In addition, an increase in sucrose concentration helped to reduce tuber size, especially tuber length; because sucrose concentration has high influence on the osmotic potential of the culture medium and further affected cell turgidity during its growth and elongation, especially in cell length. Similar to Piyatrakul (2004) reported that the highest sucrose concentration at 8 % gave significant decrease in both number of tuber per plant and tuber size in *Habenaria rhodocheila* Hance. However, Chaithanu (1999) reported that seedlings of *Habenaria rhodocheila* did not show

significant differences in number of tuber and tuber size after culturing in the modified Vacin and Went (1949) medium supplemented with sucrose at 2, 4 and 6 % in combination with paclobutrazol at 0, 0.001, 0.01, 0.1 or 1.0 mg/l.

An interaction effect of sucrose and illumination studied showed that the plantlets produced tuber in the dark condition better than in light when cultured in the medium having sucrose, while in the absence of sucrose, the tuber was produced well in the light. This is the same as in the natural habitat, where tuber is usually formed under the ground that does not expose to light. Thus, the *in vitro* culture of plantlets under the dark condition was more suitable for tuber formation and development similar to that in the natural condition. However, in the dark condition and without sucrose, the *in vitro* plantlets had insufficient carbon source for their growth and development; but when they were exposed to light, they could synthesized some sucrose to compensate the exogenous sucrose and further resulted to improve their tuber formation. In addition, plantlets cultured in the dark produced the biggest tuber in the medium supplemented with sucrose at 3 %, which was higher level than the optimal level for tuber formation in light, i.e. 2 %. In conclusion, the tubers could grow well in the dark, and required more sucrose than those growing in the light.

The coconut water concentration had no pronounced effect on tuberization percentage but in Experiment 2, the highest level tested at 20 % resulted to delay tuber formation during culturing for 2 weeks. BAP at low concentrations, i.e. 0.01 – 0.10 mg/l had no pronounced effect on tuber formation, while BAP at high concentrations from 0.5 – 2.0 mg/l distinctly affected the decrease in tuberization percentage, but when supplemented with coconut water, the BAP activity to suppress tuber formation would be interrupted, and the tuberization percentage was increased. Plant differentiation, growth and development are under simultaneous and interacting control of all plant hormones (Rappaport, 1978). An agreement with this experimental result, the BAP at high concentrations had stimulated cell division in a meristematic tissue, which should be the origin of tuber primordium; but it produced protocorm-like bodies instead of tuber formation. But an addition of the coconut water changed the balance of auxins and cytokinins, and promoted the tuber primordial development to a normal tuber formation. It has been reported on micro-tuberization of *in vitro* potato nodal segments that thidiazuron at 0.1 mg/l and kinetin at 2 mg/l delayed the onset of tuber initiation and lessened tuber production under long day photoperiod (Kefi *et al*., 2000).

For tuber growth, coconut water supported an increase in tuber size, especially in tuber width, but the highest level at 20 % showed a trend to decrease in tuber length. Because coconut water contains important complex substances that can act as growth factors (Arditti and Ernst, 1993), including cytokinin (Letham, 1974). Thus, an addition of coconut water at the highest level has increased cytokinin which promoted in cell division but negatively affected cell elongation (Rappaport, 1978), promoting an enlargement of tuber width, but reduced its length. According to Nualkaew (2004) reported that *Brachycorythis* sp. cultured in a medium supplemented with coconut water at 15 – 30 % (v/v) produced tubers bigger than those obtained from the medium devoid of coconut water, while in the presence of coconut water at different concentrations, there was no significant difference in tuber size.

Similarly, BAP at low concentrations from 0.05 – 0.10 mg/l slightly increased tuber size, but adding at higher levels at 0.5 – 2.0 mg/l, the tuber size was decreased, especially in its length. Interestingly, similar to tuber formation, when the coconut water was added, the tuber increased in size when compared to that from the same level of BAP singly used. Similar to Nualkaew (2004), BAP at 1.0 mg/l gave a decrease in *Brachycorythis* sp. tuber length, this effect was more pronounced when supplemented together with auxin NAA at 0.5 – 2.0 mg/l.

Addition of auxin IAA at 0.01 – 1.00 mg/l had no significant effects on tuberization percentage and tuber size. However, the highest level at 1.00 mg/l slightly decreased tuber length, similar to the effect of NAA on a decrease in tuber length in *Brachycorythis* sp. (Nualkaew, 2004). Because each plant tissue responds differently to different level of auxin and/or auxin, especially at high concentration acts to increase cell enlargement that supports a decrease in cell elongation. In addition, it has been reported that auxin can stimulate the production of ethylene *via* stimulating ACC synthase production (Rappaport, 1978; Wikipedia, 2007a). The ethylene has a dual effect on cell wall extensibility and inhibiting it in the longitudinal direction, but increasing it laterally, resulting in elongation inhibition and promotion of lateral expansion growth in pea epicotyls (Roberts and Hooley, 1988).

Tuber shape

A series of experiments showed that *in vitro* plantlets usually produced the oval-shape tubers when cultured in a dark condition similar to the shape obtained in nature. But culturing in light or in the media supplemented with IAA or BAP, the cultures provided decreases in percentage of the oval-shape tuber formation. It was found that light was important factor to inhibit the formation of the oval-shape tuber, and IAA and BAP enhanced the inhibitory effect. However, the effects of IAA and BAP were reduced when the plantlets were placed in the dark.

Considering *in vivo* tuber formation, a tuber is formed under ground. The growth of the tuber primordium which later produced a tuber stalk came from elongated cells at the sub-apical meristem region and the interval layer of leaf primordia. It is similar to the elongation of young shoot in the dark condition, but the direction is reverse. This physiological procedure is influenced by auxin and gibberellins (Roberts and Hooley, 1988; Wikipedia, 2007a; Wikipedia, 2007c), which are synthesized in the apical meristem and young leaf. It has been reported that the gibberellins increased in production in the dark (Wikipedia, 2007c), and enhanced the auxin effect (Wikipedia, 2007a). For the cell elongation process, auxin directly stimulates the early phases by lowering the pH around cells to break bonds in the cell wall structure (Wikipedia, 2007a), which helped to increase the elasticity of cell walls (Rappaport, 1978), and the simultaneous uptake of water by osmosis resulting in cell extension, while gibberellins play a role in the regulation of straight growth. In conclusion, both auxin and gibberellin promote the plastic extensibility of stem elongation (Roberts and Hooley, 1988).

According to the above reasons, plantlets have extended the tuber stalk normally in the dark, and formed the oval-shape tubers similar to those occurred in nature. But when they were exposed to light, the gibberellin production was decreased, which had a negative impact on the tuber stalk extension. Similarly, in

shoot growth (Experiment 5) the artificial light could inhibit the elongation of shoot and young leaf. It showed that the intensity of the artificial light used was sufficient to reduce gibberellin production. Thus, culturing in the dark condition gave better balance of growth regulators for tuber stalk extension and the oval-shape tuber formation. While in the light condition, reducing in gibberellin production changed a balance of growth regulators and then provided an equal expansion throughout the tuber stalk and tuber tip, or without oval expansion of tuber body, or a presence of short tuber stalk extension.

Adding auxin IAA stimulated cell enlargement and an increase in IAA concentration provided more effectiveness. In addition, BAP especially at higher concentrations has a stronger effect to promote cell division and also inhibit cell elongation (Rappaport, 1978). Thus, adding of IAA or BAP changed a balance of growth regulators for tuber growth and development.

The above discussions can be used to explain the growth of tuber, i.e. more lateral expansion than longitudinal extension of the tuber stalk part, and the lateral expansion (as in root swelling) of the tuber body which finally formed the cylindrical-shape tuber.

Formation of a new tuber primordium

After a plantlet had already formed the first tuber, it continued to produce a new tuber primordium closed to the position that the first tuber emerged. The histological change was studied. It showed that this bud was formed similar to the formation of an adventitious bud in general. In addition, the new tuber primordium was initiated from the tissue at the basal position of the previously formed leaf primordia, this phenomenon occurred in the tuber stalk of the primary tuber. The new tuber primordial cells divided and continued to grow until it emerged through the primary tuber stalk into the front direction of the mother protocorm and eventually formed a new young tuber bud (Figure 29).

This process is different from the natural occurrence; after the subterranean tuber has been formed, the *in vivo* plantlet enters the dormancy phase after aboveground parts die when the unsuitable season arrives, but *in vitro* plantlet got sufficiently essential growth factors from the CMU_1 medium comprises various plant nutrients and growth substances, e.g. vitamins, sucrose and coconut water. In addition, the *in vitro* culture gave suitable conditions both chemicals and physical factors e.g. temperature and humidity for the plantlets to grow continuously, and/or together with the meristematic tissue at the base of the previous leaf primordia was less influence by the apical dominant effect caused by the primary apical meristem had moved some distance downwards to form a tuber shoot bud and also included with the auxin producing by the tuber shoot bud meristem may be transported in downward direction. In accordance with the statoliths, sedimentation of amyloplasts in plant cells caused by geotropic force and plays a role in the geotropic signal that leads auxin flow into a gravity direction (Smith and Martin, 1993; Wikipedia, 2007e).

This phenomenon of the new tuber primordium formation was different from the formation of many tubers previously produced. According to this experimental result, it was found that some plantlets had simultaneously produced 2 – 3 tubers at once, which derived from the same meristem of the first tuber primordium.

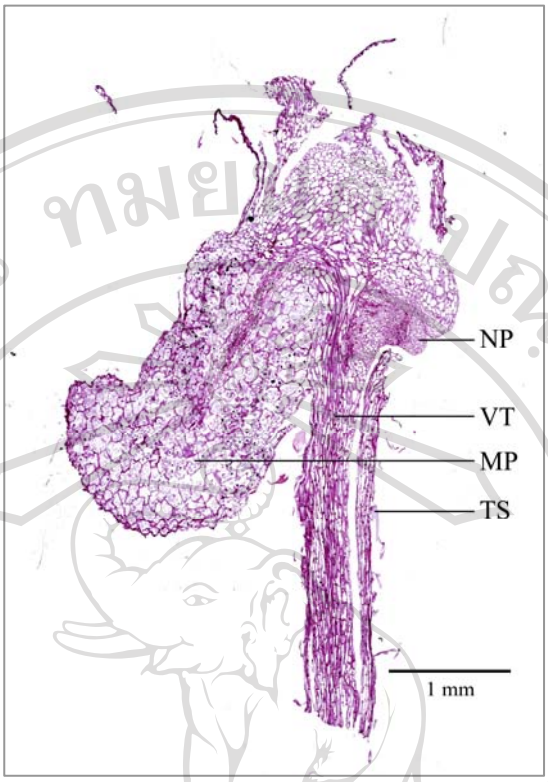

Figure 29 A longitudinal section of a seedling showing the formation of a new tuber primordium.
NP = New tuber primordium, VT = Vascular tissue, MP = Mother protocorm, TS = Tuber stalk

3. Root formation and growth

It was found that the highest osmotic potential of the culture medium supplemented with 7 % sucrose showed some trends to reduce in rooting percentage and root growth. The high osmotic potential, undoubtedly, resulted in decreasing cell water uptake which then further affected their metabolism and growth.

In addition, coconut water has no significant effect on rooting percentage and root growth. However, addition of the coconut water at 10 – 15 % gave better root growth, because coconut water contains various growth substances which promote root growth. According to other orchids, *in vitro* seedlings of *Paphiopedilum concolor* (Phornsawatchai, 1992) and *Brachycorythis* sp. (Nualkaew, 2004) provided better root growth when cultured in the CMU_1 medium supplemented with coconut water at 10 and 15 %, respectively. Furthermore, an addition of coconut water in the medium containing BAP gave better root growth than those cultured in the medium having only BAP. In addition, increased BAP concentrations at 0.1 – 2.0 mg/l reduced rooting percentage and root growth due to lowered ratio of auxin to cytokinin, which inhibits root growth and development (Rappaport, 1978; Wikipedia,

2007b). While adding coconut water provided more growth substances, and then promoted root growth and development.

As for illuminating condition, it was found that the low light intensity used reduced rooting percentage and root growth of the *in vitro* seedlings, since light can disturb auxin transportation and its regulation. Any stimulus which enhances or inhibits movement of plant growth regulators can have a significant impact on plant growth and development (Roberts and Hooley, 1988).

4. Formation of protocorm-like bodies

The cytokinin, BAP at 0.1 - 2.0 mg/l enhanced protocorm-like bodies (plbs) formation. Increasing BAP concentration together with coconut water at 15 % gave higher percentage of plantlets forming the plbs. The BAP and coconut water had interaction effect on number of plbs; the plantlets produced the highest number when cultured in the medium supplemented with only BAP at 0.5 mg/l, but when having coconut water, the used BAP level providing the same number of plbs formation was higher to 1.0 mg/l. Because of the ratio of auxin to cytokinin had affected cell differentiation and development (Rappaport, 1978), and increasing in cytokinin level promoted bud formation including the plbs formation in orchid tissue culture. An addition of coconut water promoted the tuber primordia growth which impeded the BAP effect on the formation of plbs.

For histological study on the plbs formation, it was found that the meristematic tissues of the initial protocorm had increasingly divided cells and further separated to produce several apical meristematic dots. After that, the new apical meristems continued to grow and emerged through the surface surrounding the initial protocorm and eventually formed the plbs (Figure 30).

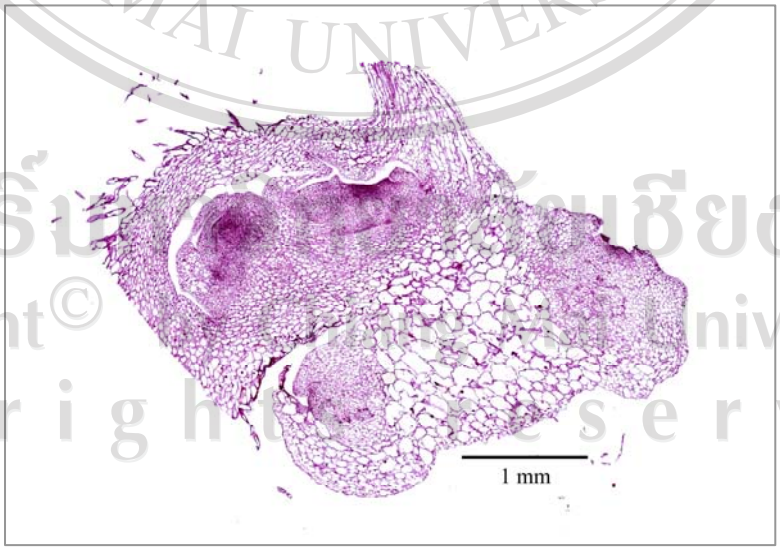

Figure 30 A longitudinal section of protocorm showed the multiple formation of apical meristematic dots.

5. Total soluble sugar and starch accumulation

5.1 *Effects of coconut water, IAA and BAP*

This experiment was carried out under a dark condition where photosynthesis could not occur to produce some photosynthetic sucrose and starch in the *in vitro* cultured plantlets. However, it has been reported that the leaf discs of de-starched tobacco plants that floated on sucrose or glucose solution in the dark could accumulate starch, while other leaf discs floating on water and being illuminated could form the photosynthetic starch (Herold, 2005). Thus, the changing in the total soluble sugar and starch contents in different plant organs might be caused by their metabolism for seedling growth and/or translocation of sucrose and/or the conversion between sugar and starch, and/or the uptake of sugar from the culture medium. In addition, each plant organ has different function and response to each level of plant growth regulators.

Coconut water had the effects to decrease the total soluble sugar (TSS) in protocorms and shoots, except in the tuber that was increased. Because coconut water stimulated the shoot growth which more sugar demand was needed, so it took some dissolved sugar from the protocorm tissue that resulted a decrease in TSS in the protocorm. While in the sink organ, tuber had increased in sugar influx from both other plant parts and the culture medium. For starch accumulation, it was found that coconut water supported an increase in starch contents in whole plant parts. It is possible that coconut water promoted the starch accumulation and/or gave higher various soluble sugars, which could be used as a precursor for the starch synthesis, e.g. glucose.

The auxin IAA had affected a decrease in TSS in the protocorms. A similar result was obtained on decreased TSS in the shoots, except when adding IAA at the highest level, 1.0 mg/l which gave an increase in TSS. In contrast, TSS in the tubers was slightly increased. It is due to adding IAA at low concentration had a trend to promote shoot growth, which was necessary to use some sugar to produce the chemical energy and other cell components, while IAA at the highest concentration had negative effect on plant growth and then resulted to decrease in sucrose catabolism. Similarly, in the tuber, IAA had influenced in reducing tuber growth and then reserved sugar content. In addition, it has been reported that auxin induces sugar and mineral accumulation at the site of its application (Wikipedia, 2007a). For the starch synthesis, IAA gave high starch contents in both protocorms and shoots. While in the tuber, IAA at low concentration supported a decrease in starch contents, but when the highest concentration was added, it gave a positive effect to increase in starch contents, due to the changing between the sugar and starch was closely related. According to Herold (2005) which had earlier been stated that the leaf discs floating on sucrose or glucose solution in the dark can accumulate starch; so that, when the plant has decreased in sucrose consumption, it will increase in starch accumulation. In the opposite, while the plant needs sucrose, the starch accumulation decreased.

Increasing BAP concentrations helped to decrease TSS in the protocorms, and also showed a similar trend in tuber. While in shoots, the TSS was slightly increased, due to BAP promoted the tuber growth that needs more energy and carbon skeleton from sugar, and also subsequently caused to translocate some sugars from

the protocorm tissue too. In contrast, BAP inhibited the shoot growth which then decreased in sugar consumption. For starch accumulation, BAP had distinctly effected to decrease in starch contents in the protocorms and tubers, but starch was increased in shoot, especially when applied at the lowest concentration, 0.01 mg/l. The same as the above reason, when the sugar level was decreased and used for tuber growth, it subsequently had an influence on a decrease in starch accumulation. In addition, it has been reported that the starch accumulation rate in sweet potato callus was sustained when cultured *in vitro* on the medium supplemented with the cytokinin kinetin at 2 mg/l, while in the absence of kinetin, the starch content in callus was diminished (Lu *et al.*, 1995).

5.2 *Effects of illumination and sucrose*

The plantlets cultured in the medium with increased sucrose concentrations had higher TSS in all plant organs, both in the dark and light conditions. In addition, the plantlets exposed to light showed a trend to increase in TSS in whole plant organs, when compared to the results from the same levels of sucrose, because higher sucrose concentrations increased chemical potential of the culture medium, together with the sucrose was non-ionized solute which is easily capable to pass through the cell membrane via the phospholipids bi-layer, depending on different pressure potential. The plantlets cultured in the light condition could have photosynthesis to produce some sugar, which helped to increase TSS in the plant tissues.

For starch accumulation, it showed a tendency that the starch was highly accumulated in the whole plant tissues when the plantlets were cultured in the medium with rising in sucrose levels, both in light and dark conditions. According to the above reason, the rising in the TSS level in plant tissues, also indicated more amounts of the sugar, glucose-1-phosphate, which is a precursor of starch synthesis. It has been reported that sucrose was more effective in inducing regulation of ADP-glucose pyrophosphorylase (AGPase), a key enzyme of starch biosynthesis. Under both dark and light conditions, sucrose fed to the detached leaves of *Arabidopsis thaliana* was found to be rapidly metabolized to hexoses and, to some starch (Sokolov *et al.*, 1998). In addition, starch was more accumulated in the protocorm and shoot tissues of the plantlets culturing in the light condition. Because leaves can synthesized starch inside the chloroplast as a transient store during its exposing to light (Kolbe *et al.*, 2005), and then gave an increase in starch contents in the leave tissues, and might be a decrease in starch degradation in the protocorm tissues. In the tuber, it had a trend that starch was accumulated in the dark more than in the light condition. Because in the dark condition, the starchy reservoirs in leaves were degraded to support their metabolism, followed by the sucrose exportation to the storage organ and then converted to starch. According to the heterotrophic organs such as potato tubers, it has been reported that the most of the incoming sucrose is converted to the starch as a long-term carbon store for reproductive growth (Kolbe *et al.*, 2005); the same reported by Khuri and Moorby (1995) that sucrose was a suitable carbon source that is easily assimilated by potato plantlets and converted to starch in developing micro-tubers. In contrast, the plantlets cultured in a sucrose-free medium had accumulated starch in the tuber better in the light condition than the dark condition, because in the condition without carbon source, the plantlets must have

photosynthesis to produce sucrose and some chemical energy for growth and development and also starch production. This phenomenon can not take place in the dark.

Part II Histological study on tuberization.

1. The first tuberization of a seedling

The histological study on *in vitro* tuberization of *Pecteilis sagarikii* was undertaken by using different stages of seedlings culturing in the artificial optimal conditions. It may give information different from the natural condition in times of seed germination, protocorm development, seedling development and also tuber formation. In addition, another *in vitro* condition that differs from this study condition in terms of seed pod age, composition of culture medium and also physical factors may cause different results. According to Rasmussen (1995), the studies on growth and development of cultivated plants can provide information on the organogenesis in nature, but the growth rate and organogenesis are probably much faster than they would be under natural conditions. The life history of cultivated material is usually shorter than the estimates predicted from the studies of excavated material. It can be radically altered by the addition of growth regulators into the culture medium.

In this study, the tuberization process of *in vitro* seedlings occurred during 22 to 32 weeks after seed sowing. In the early process, the tuber primordium was formed after the meristem had already produced some leaf primordia, which further developed to functional leaves and photosynthesized to produce the essential substances for seedling growth. In the following step, cell division occurred in the tissue between the tuber primordium and the shoot meristem, which later grew to push the tuber primordium into the opposite direction of the mother protocorm, in order to place the tuber primordium on a suitable position for continuous growth to form a tuber stalk in the downward direction. According to the natural ecological habitat, the tuber stalk serves both to adjust the depth of the plant and to colonize new soil at some distance from the mother plant (Rasmussen, 1995). In addition, the tuber primordium was found to produce some new leaf primordia covering the shoot meristem to form a new tuber-shoot bud after the tuber stalk reached an optimum depth for the daughter tuber survival in unsuitable environment during its dormancy, and also can support the new growing plant with an appropriate depth of soil. The ontogeny of the tuber stalk, being formed by the tuber primordia becoming concave to form a deep cup at the bottom of which the youngest leaf primordia of the protocorm are located to form a hollow stalk, the same as the tuber stalk formation of *Orchis mascula* (Rasmussen, 1995). In the last stage of tuber formation, the meristematic cells at the tuber tip divided to extend the tuber length and formed one to three vascular tissues or steles, together with the enlargement of cells surrounding the steles to increase tuber width, and also accumulated starchy reservoirs for survival in a dormant period and for the next growing season. The number of the steles formed might be related to the seedling health, the composition of the culture medium, and

culturing conditions. In another terrestrial orchid, it has been reported that the first tuber is formed in the young plant of *Galearis spectabilis* contains only one stele (Rasmussen, 1995).

2. The tuberization of a mature plant

The relationship of the external change of a growing shoot and the changing of its shoot tip with the tuber primordia formation and its growth in different stages of plant development were observed. The tuber primordium had been developed from the most complete adventitious bud at the basal part of the plant stem in its vegetative stage until the tuber bud was completely developed in the last period of the vegetative stage. Similar to the tuber formation in other orchidoid orchids, such as *Habenaria malintana, H. rhodocheila* (Chanaken, 2007), *Orchis, Platanthera, Dactylorhiza* and *Ophrys*, it originated as an axillary shoot bud that always formed in the axil of a leaf or scale leaf at the basal part of stem (Rasmussen, 1995). At this stage, the smallest leaf bract occurred at the central part of the plant shoot. This phenomenon can be used to indicate the transition process, i.e. internal hormone changes, from the plant vegetative stage to the generative stage which may have already been completed, although the exact changing shape of the plant apical meristem dome to a floral-spike primordium at the initial transition stage could not be detected. This phenomenon may be called the pre-flowering stage. According to Jordan (1993), the transition to flowering is the consequence of a progressive series of changes at the apex in which the indeterminate pattern of leaf and internode production is modified to produce a determinate floral structure. Two distinct phases in the transition from vegetative to floral development can be observed. First, the pattern of apical organization is altered. This may be coincide with an obvious physical change such as an increase in the apical volume. As the subtle internal forces which establish the patterns of apical self organization are modified, phyllotaxis and primordium spacing is altered. The net result of these changes is an altered three dimensional arrangement of growing points, giving rise to the inflorescence organization. In the second phase floral differentiation occurs. Internode elongation is suppressed and modified leaf structures are formed as whorls of sepals, petals, stamens and carpels. When an apex, either axillary or terminal, is fully florally differentiated, development is determinate.

In an observation that had not been shown in this study revealed that the mature plant had several buds in the axils of the upper rosette leaves. These buds could develop further into new tuber primordia if the first tuber primordium was injured or removed, e.g. while it was collected for this histological study.

Subsequently, when the 2 – 3 small leaf bracts appeared, the shoot meristem could be visualized (after removing the small leaf bracts), thereafter, differentiated into floral primordia and produced some floret buds; while the tuber primordium started to extend and continued to grow straight down to produce a tuber stalk and formed a new tuber shoot bud at the optimal depth and increased in tuber size. From the histological study, the extension of the tuber primordium originated from the dividing meristematic cells at the base of the tuber primordium to push it some distance from the mother plant and grew in the downward direction, and then the meristem including the youngest leaf primordia became concave to form a deep cup

as a hollow in the tuber stalk. The formation of the tuber stalk was, similarly, reported in other orchids such as *Orchis mascula, Orchis militaris, Orchis pallens, Aceras anthropophorum, Himantoglossum hircinum, Ophrys holoserica* and *Serapias* (Rasmussen, 1995). The length of the tuber stalk serves both to adjust the optimal depth of the new plant and to colonize new soil for the next growing season, the same as the above discussion in the *in vitro* formation. The starting growth of the tuber primordium occurs similar to the growing of a lateral bud when the apical dominance causing by the auxin action has been diminished during the transition stage from a vegetative to a floral apex, and the auxin level may be decreased and/or increasing in cytokinin. The auxin plays a minor role in the initiation of flowering (Wikipedia, 2007a), while the cytokinin breaks shoot apical dominance and promotes lateral bud growth and development (Pierik, 1977; Wikipedia, 2007b), and also induces floral bud initiation *in vitro* (Teerawatsakul, 2003).

Afterward, the floral bud started to emerge and developed into a complete floral spike until the floret buds opened, the tuber simultaneously continued to increase in size, developed a new complete tuber-shoot bud; and also accumulated some starchy reservoirs until the complete tuber was obtained. For histological study during this stage, the new tuber-shoot meristem produced some leaf primordia covering the meristem until the new tuber-shoot bud completely formed. It is possible that both the tuber stalk and multi-layers of the leaf primordia have a function to protect the youngest shoot while the tuber becomes dormant, and when the young shoot is emerging through the ground in the next growing season.

Part III Studies on the changing of internal macro elements, free amino acids and some growth regulators in mature plants during tuberization.

1. Changing in fresh weight, dry weight and water content

The plant lost dry matters during producing some leaves and roots in the vegetative stage. In this stage, the shoot just emerged from the tuber and grew rapidly, and need plenty of nutrient and energy, but it could not sufficiently obtain from photosynthesis. Hence the nutrient reservoir in the tuber was consumed, resulting in losing dry weight distinctly. In contrast, the plant in the pre-flowering stage continued to increase in fresh and dry weights in shoot, tuber and root, and also increased in total dry weight. Because the plant had completely developed leaves and roots, thence it could photosynthesize efficiently to produce some metabolic substances.

2. Analysis of macro elements

The nitrogen concentration in the tuber had increased continuously during shoot emergence and producing some leaves in the vegetative stage, but in the pre-flowering stage, the nitrogen level was slightly decreased both in tuber and shoot. Because in the sprouting stage the plant utilized some storage reservoirs in the tuber,

e.g. amino acids, an important storage form of nitrogen, and probably obtained some nutrient from its symbiotic mycorrhizal fungi (Arditti, 1992). When the leaves and roots grew and completely developed in the vegetative stage, the plant could absorb more nitrogen ion from the soil and also synthesized to produce a large quantity of nitrogenous substances, e.g. amino acids, proteins, vitamins, coenzymes, nucleic acids, ATP, chlorophyll (Arditti, 1992) and plant growth regulators, which are necessary for plant growth. The excess produced nitrogenous substances might be transported to store in the tuber as transient reservoir nitrogen that resulted increase in nitrogen level in the tuber. While in the pre-flowering stage, there was a change in internal chemicals, e.g. the plant growth regulators, to stimulate floral bud and tuber formation which required more nitrogenous substances for their activities. This may also be the reason in decreasing the ratio of nitrogen to the total dry matter of the leaves. It has been reported on relation of nitrogen level and flower induction, Arditti (1992) concluded that high nitrogen content induced rapid vegetative growth rate but not conducive to flowering, whereas increased level of phosphorus induced flowering. In addition, the results from this histological study in the pre-flowering stage, the tuber primordium started to grow and formed a new tuber. The old tuber might decline in its function as a sink organ and then turned into complete function as a source organ. Moreover, Marschner (1986) reported on a result of sink competition that root activity on nutrient uptake declined with the onset of the reproductive stage. Thus, the transient reservoir nitrogen in tuber was continuously used for plant growth and then resulted to decrease in nitrogen level. Similar to Chanaken's report, (2007), the nitrogen concentration in tubers and leaves of *Habenaria rhodocheila* and *H. malintana* had slightly decreased when grew into the pre-flowering stage.

The phosphorus concentration in tuber increased in the early sprouting stage because in this stage the plant started to utilize some storage nutrient in tuber and also increase in its metabolic pathway, e.g. glycolysis, the Krebs cycle and cytochrome oxidation to produce more energetic chemical substances, e.g. ATP and NADP, including a large number of the intermediate substances in those pathway, e.g. sugar phosphate, which need more inorganic soluble phosphates, then caused an increase in the phosphate level. Thereafter, the phosphate level in the vegetative and pre-flowering stages in both tuber and shoot slightly decreased, although the plant in these stages were rapidly growing, and need the energetic chemical substances, nucleic acids and phospholipids which were produced from phosphate. The increases in plant growth and dry matter also helped to decrease the ratio of phosphorus contents to the total of plant dry matter especially in the shoot dry weight; and/or the inorganic soluble phosphate might be used in a large quantity more than those obtained from the growing medium. The trend in changing of phosphorus concentration is, similarly, reported in *Habenaria rhodocheila* and *H. malintana* (Chanaken, 2007).

The potassium concentration in tuber had increased in the early sprouting and vegetative stages and then slightly decreased in the pre-flowering stage. Because in the early sprouting and vegetative stages, the tuber acted as an important source of the starchy reservoirs, and needs the potassium ion for the translocation of sucrose (Marschner, 1986; Arditii, 1992) to the sprouting shoot and the growing leaves in the vegetative stage especially in the early phase that need more energy, but leaves could not sufficiently to produce photosynthetic sucrose. Whilst in the pre-flowering stage, plant developed completely functional leaves which could produce sufficient sucrose

due to the tuber starchy reservoirs was decreased, and/or the tuber started to decline in its function the same as above discussion, thus the potassium demand in the tuber for sucrose exportation was decreased. While in shoot, the potassium level in the vegetative stage had higher concentration than that in the tuber and also slightly increased in the pre-flowering stage because the plant needs more potassium for the leaf activity, e.g. turgid movement of stomata and photosynthesis, and sucrose translocation to other growing points, i.e. shoot tip and a new tuber primordium which had rapidly grown. Potassium acts in cell turgor, cell enlargement, enzyme activities and protein synthesis (Marschner, 1986; Arditii, 1992). Chanaken (2007) reported that the potassium concentration in the tuber of *H. malintana* decreased, while that in the leaves of *H. rhodocheila* increased when those plants were grown into the pre-flowering stage.

As for the calcium concentration in tuber and shoot, it increased in the vegetative stage and consistently increased until the pre-flowering stage. Because the calcium is used for synthesis of middle lamella, permeability of cell membranes, chromosome movement during cell division and helps in some enzyme functions (Arditti, 1992). In the vegetative stage, the plant produces some leaves and roots which need a large quantity of calcium. The roots can absorb the calcium ion from the soil for plant growth and also translocated the excess calcium to reserve temporarily in the tuber and/or used as a component of alpha-amylase, increases the activities of phospholipases and ATPases (Marschner, 1986).

The magnesium concentrations in both tuber and shoot showed slight increases in both the vegetative and pre-flowering stage. Because magnesium is necessary for plant growth, it is an important component of plant chlorophyll and also activates many enzymes concerning in protein synthesis, ATPases and electron transfer in the photosystem II (Marschner, 1986). Thus, an increase in magnesium concentration especially in shoot during plant growth and leaf production was similar to the increase in the calcium concentration previously described.

3. Analysis of free amino acid

The analysis of free amino acid contents from various tissues in different stages of plant development showed that the asparagine was an important dominant free amino acid in whole plant tissues, and also in different stages of development. In addition, it was found that the arginine and the anserine were second dominant free amino acids in tuber, while only the arginine was a second dominance in root tissue. Similarly, in the other orchids, e.g. *Cirrhopetalum ornatissimum*, *Cymbidium aloifolium*, *Haemaria discolor* and *Dendrobium kingianum*, the asparagine showed as the dominant free amino acid in their leaf tissue. It has been reported that the arginine was a dominant free amino acid in the leaf tissue of *Phalaenopsis amabilis* (Arditti, 1992). This is due to the asparagine is an important free amino acid form which is almost used universally in plant as nitrogen storage and a transport compound. In addition, the enzyme asparagine synthetase has been isolated from the cotyledons of a number of germinating seeds (Barrett, 1985). In plant, asparagine is the major free amino acid in the long-distance transport of soluble nitrogen compound, chloride may also play an important role in nitrogen metabolism by increases the affinity and

efficiency of asparagine synthetase for the substrate glutamine to produce asparagine. Therefore, chloride is one of the major osmotically active solutes in the vacuole and thus effects, for example, the turgor potential of leaves and the stomatal movement (Marschner, 1986).

4. Analysis of free IAA and ABA contents

The analyses of free IAA and ABA contents in the tuber samples in different stages of plant development from the dormancy to the pre-flowering stage were not successful. The entire standard heavy radioisotope hormones and the sample hormones could not be detected after the analyses with GCMS. Many correctives were carried out to solve this problem but did not succeed. This may be explained that the tuber contains a large amount of amino acids and carbohydrate substances such as monosaccharides and polysaccharides, consisting mainly of mucilage and starch, and probably both the internal standard radiolabel hormones and free plant hormones might be bound with amino acids and/or glucose to form the inactive storage forms of IAA and ABA, which could not be extracted with the diethyl ether. According to Roberts and Hooley (1988), the cellular complement of plant growth regulators comprises both free molecules and forms conjugated to sugar or amino acid molecules. Therefore, the extraction methods should be sought to restrict both the hydrolysis of conjugates and the conjugation of free plant growth regulators. To date, considerations of this conversion have been rare.

However, the concentration of free IAA and ABA in the shoot could be measured both in the vegetative and the pre-flowering stage. It was found that the concentrations of IAA and ABA had declined from the vegetative stage to the pre-flowering stage. According to the results from the previous histological study on tuber formation, this is because in the pre-flowering stage, the plant had initiated the floral bud and also the tuber primordia, growth and development; thus, decreasing in IAA levels enabled the tuber primordia to be released from the apical dominance effect, the same as the occurrence in the lateral shoot formation (Pierik, 1977; Arditti, 1992). Decreasing in auxin : cytokinin ratio can also promote the floral bud initiation. Arditti (1992) informed that auxin inhibits the flowering of monopodial orchids such as *Phalaenopsis schilleriana* and *Aranda* hybrids. Removal of their apical meristems, major sources of auxin or application with a cytokinin BAP have resulted in flower-bud initiation in *Aranda* Deborah. According to Roberts and Hooley (1988), the balances of plant growth regulators play roles in developmental phenomena, which involve the transition from one state of differentiation to another. While ABA is an endogenous plant growth inhibitor that plays a major role in plant dormancy; thus, a decline in ABA levels helped to raise the effectiveness of other plant growth regulators, i.e. auxins, cytokinins and gibberellins, to promote plant growth and development.