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Effect of Liquinox and Rosasol-N fertilizers on vegetative growth of phalaenopsis seedlings after transplantation into *ex vitro* conditions

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Abstract

Initial period of growth, under *ex vitro* conditions, is critical for seedlings previously micro propagated *in vitro* since, during this period, the seedlings are depended only upon slow photosynthetic leaves and encounter various stress factors. *Ex vitro* experiments were conducted to study the effect of NAA containing fertilizer (Liquinox) and high N containing fertilizer (Rosasol-N) on growth rate, chlorophyll content and viability of phalaenopsis seedlings previously micro propagated *in vitro*. Treatments were performed using factorial design with two factors and three levels. This study found that during 46 days period of growth in *ex vitro* conditions, seedlings added Rosasol-N underwent both substantial decreases in growth rate and chlorophyll contents. In Liquinox added plants, whilst growth rate was slightly decreased, chlorophyll content was found substantially decreased. Minor decrease of growth and chlorophyll content was found in seedlings fertilized with mixed half strength Liquinox and Rosasol-N. At day 132 after transplantation, control plants were showing the highest sugar content and viability. Collectively, these evidently show that development of phalaenopsis seedlings during initial period of growth in *ex vitro* conditions was sensitive to the addition of inorganic fertilizers. However, prolonged application of NAA containing fertilizer repressed photosynthesis via inhibition of chlorophyll biosynthesis.

Keywords: Phalaenopsis; *Ex vitro*; NAA containing fertilizer; High N containing fertilizer; Growth rate; Chlorophyll; Viability.
Abbreviations: FW_Fresh weight, NAA_α-Naphthaleneacetic acid, Liq_Liquinox, Ros-N_ Rosasol-N, P1_treatment 1, P2_treatment 2, etc., Viab_Viability.

Introduction

Seedlings, previously micro propagated *in vitro*, is regarded as sensitive to *ex vitro* conditions attributed by their abnormality in structure and functions (Triques et al., 1997; Van Huylenbroeck et al., 1998; Robinson et al., 2009; Preece, 2010; Kumar and Rao 2012; Debt and Imchen, 2010). During initial growth, after transplantation into *ex vitro* conditions, these seedlings are remains mixotrophic (Hazarika 2002, Kumar and Rao 2012) and various reports has revealed that photosynthetic activity is slow during this period. For examples, in Dinophyceae, Adolf et al., (2006) found that cellular photosynthetic performance was 24-52% during mixotrophic relative to the performance detected during autotrophic growth. Poor photosynthetic activity was also reported in plants micro propagated *in vitro* attributed by a continuous supply of exogenous sucrose (Triques et al., 1997; Hazarika 2002; Kubota et al., 2001). These slow photosynthetic activities have been considered to be the cause of low survival and growth rate after transfer into *ex vitro* conditions (Preece and Sutter 1991 as cited by Kubota et al., 2001). During growth in *ex vitro* environment, supply of exogenous sugar is not further available and plant growth is depended only upon slow photosynthetic leaves. According to Van Huylenbroeck et al., (1998), high photosynthetic activities could then occur in the plants when new leaves formed during *ex vitro* condition has attained full expansion. However, since in Phalaenopsis plants a new leaves is usually emerge every six weeks (Blanchard et al., 2007), duration of plants underwent critical period could be about 40 days. During this relatively long critical period, seedlings require a special care particularly because poor photosynthetic activities make the seedlings susceptible to pathogen (Roy et al., 2013). Furthermore, during this period, the plants also encounter various stress factors. Thus, in order to increase growth and minimized plant loss after transfers into *ex vitro* conditions, photosynthetic activities and alleviation of stress is seem to be an important factors to enhance. In plants, auxin or nitrogen are both well-known compounds that could enhance photosynthesis and mitigate abiotic stress. Whereas auxin has long been known as growth regulator that could increase photosynthetic activity (Bidwell and Turner 1966), about 75% of cellular nitrogen is located in mesophyll chloroplast and play an important role in the synthesis of various macromolecules, such as nucleic acid, amino acid, protein, amide, etc. (Hortensteiner and Feller 2002, Taiz and Ziegler 2002). Both growth regulator and nitrogen compound had also been reported to play an important role in alleviating stress. The growth regulator mitigates stress factors by signalling the induction of normal energy and metabolic flow as well as normal function and adaptation respond (Suzuki et al., 2012; Robert and Friml, 2009; Mattsson et al., 1999; Scarpella et al., 2010). Auxin is one of various growth regulators that have important role in alleviating abiotic stress (Pasternak et al., 2005; Du et al., 2012, Barreto and Nookaraju, 2007). Abiotic stress in plants could also be alleviated by nitrogen oxide which is act as signal for various physiological processes (Handcock, 2012; Popova and Tuan, 2010; Hasanuzzaman et al., 2010). This nitrogen oxide was thought to be metabolized from nitrate or nitrite (Planchet and Keiser, 2006; Popova and Tuan, 2010). In other report, abiotic stress was mitigated by addition of NO_3^- in high doses (Aragao et al., 2012). According to these authors, consumption of high energy during reduction of nitrate by nitrate and nitrite reductase in cytosol decreases the amount of excess energy release by mitochondria. Thus, addition of auxin and high nitrogen fertilizer very likely could enhance photosynthesis and mitigate abiotic stress in Phalaenopsis seedlings previously micropropagated *in vitro*. So, addition of whether auxin containing fertilizer or high N containing fertilizer very likely could promote high growth and viability of seedlings after transplantation into *ex vitro* conditions. Accordingly, this study was then performed to ascertain whether application of auxin containing fertilizer (Liq) or nitrogen rich nutrient solutions (Ros-N) could enhance growth of Phalaenopsis seedlings after transplantation into *ex vitro* conditions.

Results

Effect of non-mixed fertilizer on growth parameters of Phalaenopsis seedlings during the first 46 days growth period in ex vitro conditions

After transplantation into *ex vitro* condition, Phalaenopsis seedlings responded differently to the addition of Liq and Ros-N. During 46 days period after transplantation, growth rate was 15.03 mg plant^{-1} day^{-1} (Tab. 1) in plants continuously fertilized with full strength Liq (P3), accounted for 122% relative control plants (Fig.1). By contrast, growth rate of plants fertilized with full strength Ros-N (P7) was only 5.29 mg plant^{-1} day^{-1}, accounted for about 43% relative control. During this periods, whereas the amount of chlorophyll-a detected in plants fertilized with full strength Liq (P3) was significantly lower than that detected in plant fertilized with Ros-N full strength (P7, Fig. 2), chlorophyll b was significantly higher in plant fertilized with full strength Liq relative to plants fertilized with full strength Ros-N (Fig. 3). In both treatments, total chlorophyll content was almost similar (Fig. 4). Thus in the mixotrophic period, although total chlorophyll content is almost similar, Liq enhanced accumulation of fresh weight in Phalaenopsis seedlings, but Ros-N inhibited this process. Similar to that found in plants added full strength fertilizer, growth rate of plants fertilized with half strength Liq (P2) was also higher than that fertilized with Ros-N (P4). In Liq added plants, growth rate was 9.62 mg plant^{-1} day^{-1} (Tab.1) accounted for ca 78% relative control (Fig.1). By contrast, plants fertilized with half strength Ros-N was showing growth rate only 2.37 mg plant^{-1} day^{-1} accounted for ca 19% relative control. Whilst, chlorophyll-a content in plants added half strength Liq was significantly lower than in plants added half strength Ros-N (Fig.2), chlorophyll-b and total chlorophyll content was almost similar (Fig.3, 4). This finding was confirming the possibilities that during mixotrophic period, Liq enhance the accumulation of fresh weight but Ros-N inhibit this process. Furthermore, since chlorophyll content detected in the plants was almost similar, enhancement of fresh weight accumulation by Liq was possibly occurred via promotion of normal metabolic and cell function by the NAA containing nutrient solution.

Effect of mixed fertilizers on growth parameters of Phalaenopsis seedlings during the first 46 days growth period in ex vitro conditions

Unlike plants added non mixed fertilizer where growth rate was enhanced although chlorophyll content was similar, addition of mixed fertilizer showed slightly different responds. When mixed half strength Liq and Ros-N was added (P5), growth rate was 9.59 mg plant^{-1} day^{-1} (Tab.1) accounted for about 77.9% (Fig.1). However, when plants were fertilized with mixed full strength Liq and Ros-N (P9), growth rate was very low (Tab. 1) accounted for less than 5% (relative control). These observed growth rate was comparable to chlorophyll content detected in the plants where chlorophyll content was significantly higher in mixed half strength fertilizer than that found in plants fertilized with mixed full strength fertilizer. Phalaenopsis seedlings added half-strength mixed fertilizer (P5) was showing 1.4, 2.1 and 3.6 mg/l chlorophyll a, b and total chlorophyll, but plant added full strength mixed fertilizer (P9) was showing only 1.2, 1.5, 2.8 mg/l chlorophyll a, b and total chlorophyll (respectively). In other cases, growth rate of Phalaenopsis seedlings, fertilized with mixed half strength Liq and full strength Ros-N (P8) was 2.84 mg plant^{-1} day^{-1} accounted for about 23% (relative control). This growth rate was much lower to that found in plants added with mixed full strength Liq and half strength Ros-N (P6), i.e. 8.26 mg plant^{-1} day^{-1}. In both P8 and P6 plants, chlorophyll content was almost similar (Tab.1). Thus, during the first 46 day period after transplantation, this experiment is consistently showed that NAA containing fertilizer (Liq) enhanced growth rate of Phalaenopsis seedlings. By contrast, these seedlings were very sensitive to the addition of high N containing complete nutrient solution, albeit chlorophyll content in these seedlings was almost similar. Implying that chlorophyll did not normally function in leaves previously formed during *in vitro* condition but could then be normalized by NAA containing fertilizers.

Effect of fertilizers on growth parameters of Phalaenopsis seedlings after the first 46 days growth period in ex vitro conditions

Unlike evident found in the initial period, addition of Liq and Ros-N into Phalaenopsis seedlings in later period was showing an opposite result. At day 132 after transplantation, sugar content was not detected in plants continuously fertilized with full strength Liq (P3), full strength Liq and half strength Ros-N (P6), full strength Liq and full strength Ros-N (P9). However, sugar content was detected in plants continuously fertilized with Ros-N (Tab. 1). For example, Phalaenopsis seedlings added solely with full strength Ros-N was showing 0.02 (P7) and plants added half strength Ros-N was showing 0.03 mg/g FW sugar (P4). By contrast, Phalaenopsis seedlings continuously added full strength Liq whether mixed or non-mixed fertilizer was not showing a detectable sugar content. Thus, leaves of Phalaenopsis seedlings emerged under *ex vitro* condition was sensitive to the addition of NAA containing nutrient solution (Liq), but did not sensitive to the addition of high N containing complete nutrient solutions (Ros-N).

Effect of fertilizers on viability of Phalaenopsis seedlings during growth period in ex vitro conditions

Among those growth parameters, the most critical parameter for field practice was the number of plants still maintaining

Table 1. Growth parameter of Phalaenopsis seedlings after transplantation into *ex vitro* condition. Means with the same letters do not different significantly at $p \leq 0.05$.

Treat.	Fertilizers		Growth rate	Chlorophyll (mg/l)			Sugar	Viab.
	Liq. (ml/l)	Ros-N (g/l)	(mg/plant/day) Mean±SD	a Mean±SD	b Mean±SD	Total Mean±SD	(mg/gFW)	(%)
P1	0	0	12.30±29.00^{a}	1.47±0.04^{d}	2.18±0.04^{e}	3.64±0.06^{e}	0.75	100
P2	2.5	0	9.62±24.55^{a}	1.25±0.03^{b}	1.79±0.06^{d}	3.04±0.08^{c}	0.16	90
P3	5	0	15.03±14.02^{a}	1.12±0.04^{a}	1.64±0.04^{bc}	2.75±0.02^{a}	-0.12	80
P4	0	1.5	2.37±24.02^{a}	1.39±0.03^{c}	1.78±0.03^{d}	3.16±0.06^{d}	0.21	80
P5	2.5	1.5	9.59±9.77^{a}	1.47±0.03^{d}	2.17±0.02^{e}	3.63±0.02^{e}	0.18	90
P6	5	1.5	8.26±25.80^{a}	1.34±0.01^{c}	1.70±0.04^{c}	3.03±0.05^{c}	-0.08	70
P7	0	3	5.29±15.35^{a}	1.24±0.02^{b}	1.53±0.02^{a}	2.76±0.02^{a}	0.02	40
P8	2.5	3	2.84±31.58^{a}	1.34±0.04^{c}	1.67±0.03^{c}	3.00±0.02^{c}	0.54	40
P9	5	3	0.50±18.45^{a}	1.27±0.02^{b}	1.58±0.01^{a}	2.85±0.02^{b}	-0.12	50
			46 day period (Mixotrophic)				Autotrophic	

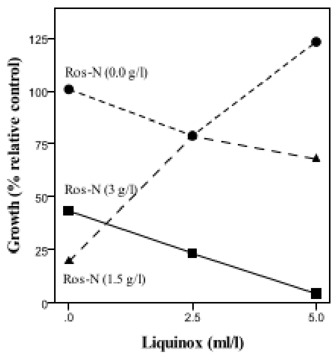

Fig 1. The growth of Phalaenopsis seedlings after addition of solution containing Ros-N, Liq or mixed solution containing Ros-N and Liq. ●, seedlings grown without application of Ros-N; ▲, seedlings fertilized with half strength (1.5 g/l) Ros-N without or with addition of Liq; ■, seedlings fertilized with full strength (3 g/l) Ros-N without or with addition of Liq.

growth after transplantation into *ex vitro* conditions. During initial period under *ex vitro* conditions, Phalaenopsis seedling was very sensitive to the addition of full strength fertilizer (Fig. 5). Seedlings continuously added full strength Ros-N was showing 70% viability and seedlings added full strength Liq was showing 80%. When plants were added half strength Liq or Ros-N, viability was increased into up to 100%. Interestingly, plants continuously grown without addition of fertilizer (P1) were showing 100% viability. In a prolonged period, plants loss was continuously occurred in plants added fertilizer but this loss did not occur in control plants. At day 162 after transfer into *ex vitro* conditions, Phalaenopsis seedlings continuously added full strength Ros-N was showing only 50% or less viability but seedlings added half strength fertilizer was showing 70 to 90% viability (Tab. 1). Seedlings that unable to maintain growth was initially exhibited symptom of soft rot (Fig. 6).

Discussions

Decreasing growth found in seedlings added nitrogen rich nutrient solution during the initial period, most likely attributed by minor ability of the plants to metabolize inorganic compounds. This is not surprising since at this period these plants had abnormal anatomy, physiology and also remain mixotrophic (Huylenbroeck et al., 1998; Kumar and Rao, 2012). In this trophic, seedlings did not find exogenous inorganic nutrient as a main nutrient source for growth. This is particularly evidenced by the high growth; viability and chlorophyll content in control plants, but did not in seedlings added nitrogen rich nutrient solutions. This implying that exogenous inorganic nutrient was not metabolized extensively in seedlings previously micro propagated *in vitro*. Leaves of seedlings with open stomata and thin cuticles layer (Robinson et al., 2009; Preece, 2010; Kumar and Rao, 2012) can easily absorb nutrient solution. Therefore, after transplantation into *ex vitro* condition when humidity surrounding the leaves then decrease, waters in the leaf then easily evaporated into the atmosphere. Inorganic nutrient accumulated in the leaves causing a quick drop of water potential. This low water potential could subsequently squeezes water from metabolic pools and inhibit metabolic machinery in the plants. These physiological problems might be the main cause of physiological symptom (Fig. 6) found in seedlings added full strength Ros-N and resulted in the decrease of growth, viability and chlorophyll contents. So, high nitrogen containing nutrient solution (Ros-N) did not mitigate abiotic stress in Phalaenopsis hybrid during initial growth in *ex vitro* environment. This evidences is therefore in variant with that reported by Aragao et al., (2012) in which high nitrogen could mitigate abiotic stress or synthesize into nitrogen oxide to alleviate abiotic stress (Planchet and Keiser, 2006; Popova and Tuan, 2010). In a moderate inorganic accumulation, NAA containing nutrient solution (Liq) could mitigate physiological symptoms. With addition of mixed solution containing half strength Liq and Ros-N, plants growth was increasing from less than 25% into about 75% (Fig.1), viability was increased into 100% (Fig.5) and chlorophyll content into a level similar to control plants (Fig.4). Implying that exogenous growth regulator NAA

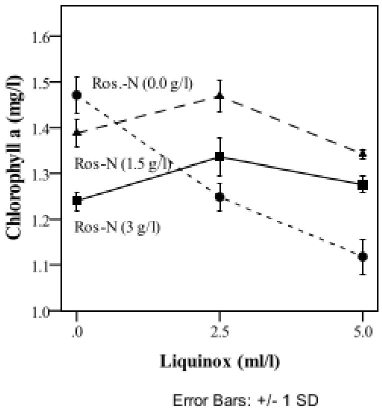

Fig 2. Chlorophyll a detected in leaves of Phalaenopsis seedlings after grown in *ex vitro* condition for 46 days. ●, seedlings grown without application of Ros-N; ▲, seedlings fertilized with half strength (1.5 g/l) Ros-N without or with addition of Liq; ■, seedlings fertilized with full strength (3 g/l) Ros-N without or with addition of Liq.

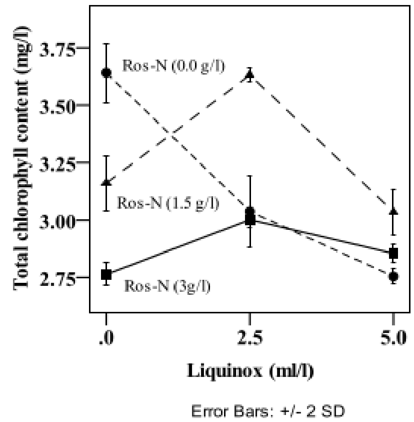

Fig 4. Total chlorophyll content detected in leaves of Phalaenopsis seedlings after grown for 46 days in *ex vitro* conditions. See also legend of Fig 2.

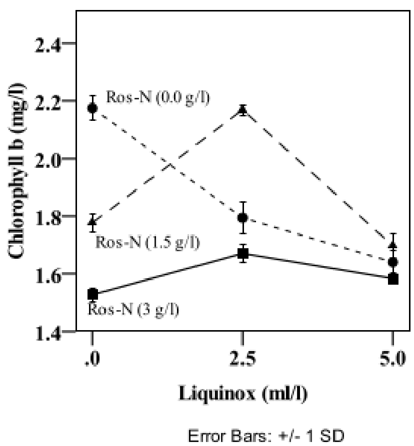

Fig 3. Chlorophyll b detected in leaves of Phalaenopsis seedlings after grown in *ex vitro* condition for 46 days. See also legend for figure 2.

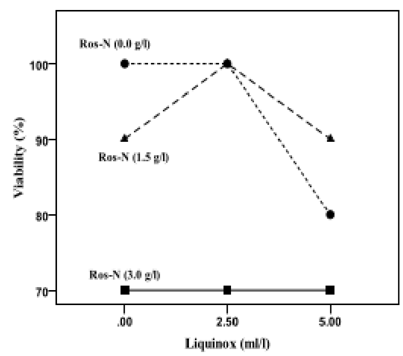

Fig 5. Viability of Phalaenopsis seedlings during growth in *ex vitro* conditions after addition of Ros-N, Liq or mixed solution containing Ros-N and Liq. ●, seedlings grown without application of Ros-N; ▲, seedlings fertilized with half strength (1.5 g/l) Ros-N without or with addition of Liq; ■, seedlings fertilized with full strength (3 g/l) Ros-N without or with addition of Liq.

could improve metabolic and cell function after accumulation of inorganic fertilizer. This evidence is in agreement with previous report that growth regulator mitigates stress factors by signalling the induction of normal energy and metabolic flow as well as normal function and adaptation respond (Suzuki et al., 2012; Robert and Friml, 2009; Mattsson et al., 1999; Scarpella et al., 2010). However, Liq was unable to alleviate an overwhelming accumulation of inorganic nitrogen, after the addition of full strength Ros-N, possibly because water potential in cell is at sub optimal level and had ceased normal metabolic and cell function. Whilst in slower rate of Ros-N application, induction of normal metabolic and cell functions by NAA increased metabolic pools and improve assimilation of nitrogen into compounds require for growth.

Fig 6. Symptom of soft rot observed in Phalaenopsis seedlings after transfer into *ex vitro* conditions. The number of plants showing this symptom was very high in Phalaenopsis seedlings continuously fertilized with full strength Ros-N.

This nitrogen fertilizer could then be synthesized into chlorophyll (Fig.2) or utilized by plants to maintain normal osmotic pressure. Implying that in a slow rate of fertilizer application, nitrogen fertilizer activated auxin signaling for nitrogen acquisition as proposed by Kiba et al., (2011). In such a way, nitrogen fertilizer was then can be synthesized into compounds require for plant growth or into compounds that functional in mitigating abiotic stress.

Materials and Methods

Plants materials and growth conditions

Phalaenopsis seedlings in bottles were purchased from a nursery in Denpasar, Bali. Each bottle contains 20 to 25 seedlings and a total of 90 seedlings were used in this present experiment. These seedlings were transferred into *ex vitro* condition after its bottles were broken in paper wrapped culture bottles. Culture media remain attached in the seedlings were washed with tap water in a container and water attached to the seedlings were then absorbed by placing the seedlings on ordinary used newspaper for about an hour. These seedlings were then grouped into 9 batches and each seedlings in the batch were then weight for it fresh weight for time zero using MA-100A mini pocket scale (ACIS). After transplantation into transparent plastic pots containing moss growth medium, these seedlings were then placed into 9 trays and then mounted into an *ex vitro* growth conditions. In this experiment, one seedling was grown in one pot (5 and 8 cm bottom and top diameter) and each tray contains 10 pots. Air humidity and dark-light regimes were natural, light intensity was reduced using shading net. These seedlings were then weighed at the second time 37 days after transplantation by lifting from growth medium.

Application of nutrient solution into the seedlings during growth in ex vitro conditions

NAA containing liquid fertilizer (Liquinox Start Grow, a product of USA) and nitrogen rich fertilizer (Rosasol–N, made in Belgium) were purchased from an Orchid nursery. Liq fertilizer had the following composition: 0.10 % Vitamin B1, 0.04 % α-Naphthaleneacetic Acid, 2 % P_2O_5, 0.10 % Fe, 90.90 % inert ingredient. The other fertilizer Ros-N had the following composition: 29 % total nitrogen, 10 % P_2O_5, 10 % K_2O, 3 % MgO, 5 % SO_3, 0.010 % B, 0.0075 % Cu, 0.026 % Fe, 0.032 % Mn, 0.023 % Zn. Liq fertilizer was applied in 3 levels, i.e. 0 (control), 2.5 ml/l and 5 ml/l and Ros-N were also applied in 3 levels, i.e. 0 (control), 1.5 g/l and 3 g/l. Combination of those two factors resulted in a total of 9 treatments as shown in Table 1. Those nutrient solutions were applied into 9 batches of plants using hand sprayer everyday commenced just after the time of transplantation until the experiment was terminated at day 162 after transplantation. In order to minimize accumulation of excess fertilizer in the growth medium, the seedlings were leached with tap water at least once a week.

Measurement of chlorophyll content

Chlorophyll content was measured 46 days after seedlings were transplanted into *ex vitro* growth conditions. Following the methods of Wintermans and De Mots as described by Suyitno (2006), chlorophyll in leaves no. 2, from each treatment, was extracted using ethanol 96% as such before measurement using UV-Vis Spectrophotometer PD-303S in triplicates.

Measurement of total sugar content

Sugars in leaves of plants were extracted using modified methods described by Foley et al., 1992. At day 132 after transplantation, leaves of plants were sampled ca 3 gram from each treatment. These leaves were incubated separately for 48 h in 20 ml 80% ethanol, before filtered using Whatman 40 filter paper. Pellet of this samples were added 5 ml ethanol 80%, grown in mortar and filtered using Whatman 40 filter paper. Filtrates from each sample were collected before heated for evaporation of ethanol on a hot plate. Volume of extract obtained after evaporation were adjusted into 3 ml by addition of aquadest. Sugar content in this extract was then measured quantitatively using Anthron methods as described by Apriyanto et al., 1989.

Conclusion

Under experimental conditions, a hybrid of Phalaenopsis seedlings was found very sensitive to the application of complete inorganic nutrient solutions Ros-N, particularly in the initial period after transferred into *ex vitro* conditions which is regarded as mixotrophic period. Addition of NAA containing nutrient solution (Liq) could mitigate the effect, but this fertilizer did not promote growth higher than control plants. However at the later period which is regarded as autotrophic, NAA containing nutrient solution Liq inhibited sugar synthesis.

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