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Low humic acids promote in vitro lily bulblet enlargement by enhancing roots growth and carbohydrate metabolism*#

Yun WU[1], Yi-ping XIA[†‡1], Jia-ping ZHANG[1], Fang DU[1,2], Lin ZHANG[1], Yi-di MA[1], Hong ZHOU[1]

([1]*Department of Horticulture, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310058, China*)

([2]*College of Horticulture, Shanxi Agricultural University, Taigu 030800, China*)

[†]E-mail: ypxia@zju.edu.cn

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Abstract: Bulblet development is a problem in global lily bulb production and carbohydrate metabolism is a crucial factor. Micropropagation acts as an efficient substitute for faster propagation and can provide a controllable condition to explore bulb growth. The present study was conducted to investigate the effects of humic acid (HA) on bulblet swelling and the carbohydrate metabolic pathway in *Lilium* Oriental Hybrids 'Sorbonne' under in vitro conditions. HA greatly promoted bulblet growth at 0.2, 2.0, and 20.0 mg/L, and pronounced increases in bulblet sucrose, total soluble sugar, and starch content were observed for higher HA concentrations (≥2.0 mg/L) within 45 d after transplanting (DAT). The activities of three major starch synthetic enzymes (including adenosine 5'-diphosphate glucose pyrophosphorylase, granule-bound starch synthase, and soluble starch synthase) were enhanced dramatically after HA application especially low concentration HA (LHA), indicating a quick response of starch metabolism. However, higher doses of HA also caused excessive aboveground biomass accumulation and inhibited root growth. Accordingly, an earlier carbon starvation emerged by observing evident starch degradation. Relative bulblet weight gradually decreased with increased HA doses and thereby broke the balance between the source and sink. A low HA concentration at 0.2 mg/L performed best in both root and bulblet growth. The number of roots and root length peaked at 14.5 and 5.75 cm, respectively. The fresh bulblet weight and diameter reached 468 mg (2.9 times that under the control treatment) and 11.68 mm, respectively. Further, sucrose/starch utilization and conversion were accelerated and carbon famine was delayed as a result with an average relative bulblet weight of 80.09%. To our knowledge, this is the first HA application and mechanism research into starch metabolism in both in vitro and in vivo condition in bulbous crops.

Key words: Bulblet development, Humic acid, Starch metabolism, Source-sink conversion, *Lilium* Oriental Hybrids 'Sorbonne'

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1 Introduction

Flower bulbs represent a significant segment of the global floriculture industry, and have a variety of ornamental uses (Benschop *et al.*, 2010). Unfortunately, due to low natural bulb multiplication rates or long juvenile phases, commercial release of a new genotype may take 10 or even 20 years by conventional vegetative propagation methods (de Klerk, 2012). A bulb is the core center of bulbous plants, and the specialized scales (modified petioles) can serve as the carbon store during the early developmental stage, providing the plants with carbon skeleton and energy for reproductive growth, then inflorescence formation and blooming, and completing their life cycle (Fig. 1 takes the lily as an example). Additionally, a larger

‡ Corresponding author

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ORCID: Yun WU, http://orcid.org/0000-0002-1612-5620

Fig. 1 Whole life cycle of commercial lily cultivar *Lilium* Oriental Hybrids 'Sorbonne' in Zhejiang Province
(a) Bulb (modified petioles, white part) with bud (the light pink part) dormancy release; (b) Sprout after planting; (c) Vegetative growth; (d) Immature flower bud; (e) Pink and fragrant flower; (f) Plant after blossom (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

bulb usually produces more vigorous plants with higher flower quality (Chen *et al.*, 2000). Speeding up the propagation cycle converts into the question of how to shorten the bulb development process and obtain a larger storage organ.

Micropropagation is a sine qua non for breeding in the present-day because of faster propagation rates in vitro, e.g., newly bred cultivars can now be introduced onto the market within 7–8 years for the lily (de Klerk, 2012), and offers many other advantages (Podwyszyńska, 2012). However, for most major bulbous crops, the protocols are still not workable for commercial use because of low propagation rates even in vitro. Plant growth regulators (PGRs) are involved in nearly all aspects of plant growth and development including in vitro storage organ formation of ornamental geophytes (Ascough *et al.*, 2008). Although many artificial PGRs have been applied successfully in bulbous crops in vitro (Gerrits and de Klerk, 1992; Marinangeli and Curvetto, 1997; Kumar *et al.*, 2005; Jásik and de Klerk, 2006), almost all reports focused only on optimization of culture conditions and work on the related bulblet growth mechanism is scarce. Among these PGRs, humic acids (HAs), complicated mixtures of biologically transformed organic debris (Hayes and Clapp, 2001), proved to be beneficial to plant growth and productivity by influencing, directly or indirectly, several plant biochemical processes (Zandonadi *et al.*, 2007). One aspect is to promote root development to be advantageous for nutrition uptake (Canellas *et al.*, 2002; Zandonadi *et al.*, 2007). Fulvic acid increased the extra-large tubers by being sprayed on the leaves of potato plants (Suh *et al.*, 2014). Argüello *et al.* (2006) also found that the use of vermicompost as a substrate caused early bulbing and lengthened the bulb filling period in garlic. To our knowledge, there are no reports on HA application focusing on the effects on bulb formation and development in bulbous plants. We have previously shown that HAs can

enhance plant growth and root development in the Oriental lily under open fields (Chang *et al.*, 2012). Thereby, we hypothesize that HAs can be beneficial to bulblet swelling in vitro.

Bulblet formation and development (also called tuberization) is a highly coordinated morpho-physiological process under the influence of both extrinsic and intrinsic factors (Podwyszyńska, 2012). Generally, large amounts of carbohydrates accumulate during bulb formation (Kawa and de Hertogh, 1992), e.g., starch constitutes the dominant storage reserve polysaccharide (about 20%–30% fresh weight (FW)). Starch, comprised of amylase and amylopectin, is an insoluble polyglucan produced by starch synthase (SS) using adenosine 5'-diphosphate glucose (ADPG) as the sugar donor molecule (Bahaji *et al.*, 2014). Granule-bound starch synthase (GBSS) can specifically elongate amylose (Nelson and Rines, 1962) while soluble starch synthase (SSS) is exclusively involved in amylopectin biosynthesis (Streb and Zeeman, 2012). We have previously reported that bulb formation and swelling are closely correlated with carbohydrates manipulation between aboveground and underground parts either in the lily or *Lycoris* under open field conditions (Zheng *et al.*, 2011; 2012; Chang *et al.*, 2013; She *et al.*, 2014). Comparative carbohydrate-related genes involved in bulblet formation and development were examined in *Lilium davidii* var. *unicolor* very recently demonstrating the fundamental role of carbohydrate metabolism, especially starch and sucrose metabolism, in lily bulblet emergence and development at the transcriptional level (Li *et al.*, 2014). The main aspects of bulb development can be agreed upon as they relate to the starch metabolic pathway. Leaves can fix carbon during the day and then remobilize the carbohydrates during the subsequent night to support photosynthetic metabolism and growth. Therefore, leaves are regarded as photosynthetic tissues whilst storage organs, e.g., bulbs, are heterotrophic organs (Bahaji *et al.*, 2014). Source-sink changes constantly in the lily plant (Wu *et al.*, 2012) and intensification of sink and source performances could enhance the yield of the storage organ, e.g., potato (Katoh *et al.*, 2015). Fig. S1 shows the classical starch metabolism and conversion model within whole flower bulbs.

In the current paper we selected *Lilium* as our model material for its high reproductive efficiency compared with the tulip or *Lycoris* (Alderson *et al.*, 1982; Kuijpers and Langens-Gerrits, 1997; Chang *et al.*, 2013). We focused solely on starch metabolism linked to autotrophic organ carbon reserve conversion. Principal carbohydrates and starch enzymes involved in this pathway were examined for different levels of HA treatment or non-HA treatment during in vitro bulblet swelling. Thus, our research can provide a fundamental theory for HA usage and for shortening bulbous plants' breeding programme. So far, this is the first application of HA in a scheme for mass propagation of not only lily but also all bulbous crops.

2 Materials and methods

2.1 Plant materials

'Sorbonne' lily bulbs (16 to 18 cm in circumference) were collected from a commercial supplier (Zhejiang Hongyue Seed Co., Ltd., China) imported from the Netherlands on September 10, 2011. The lily bulbs were immediately repacked into a plastic bag with tiny holes for cold storage in a refrigerator (4 °C) for 2 to 3 weeks before tissue culture. Individual shoots (shoot length 1–2 cm, basal diameter 3–5 mm) were obtained from induction culture for further use. The plantlets were illuminated with a 12 h/12 h photoperiod (60 μmol/(m^2·s) cool white fluorescent irradiance) at (24±2) °C in all experiments at the Physiology & Molecular Biology Laboratory of Ornamental Plants and Tissue Culture Laboratory of Ornamental Plants in Zijingang Campus, Zhejiang University, China (E 120°11′, N 30°29′) unless indicated otherwise.

2.2 Humic acid treatments and morphological observation

In total, there were four treatments, the basal MS (Murashige and Skoog, 1962) medium plus 8 g/L agar and 70 g/L sucrose as the control (CON) and the control medium containing different concentrations of HA (Aladdin®, fulvic acid ≥90%, H108498, China) doses, the low concentration HA (LHA), moderate concentration HA (MHA), and high concentration HA (HHA), with the values of 0.2, 2.0, and 20.0 mg/L, respectively. The HA was directly added to the medium prior to autoclaving. Each glass conical flask (6.5 cm diameter×10 cm height, Shuniu, China) was

planted with five sterile shoots, and a total of 200 shoots were initially used for each treatment. We harvested and observed the plantlets every 15 d until the materials were exhausted. The specific sampling dates were as follows: February 2, February 17, March 4, March 19, and April 3 in 2013 at 9:00 a.m. (Beijing Time) each day. We defined these harvesting time points as DAT15, DAT30, DAT45, DAT60, and DAT75, respectively. DAT was short for days after transplanting.

Seven morphological indices were observed to evaluate in vitro bulblet growth: plantlet height, number of leaves, number of roots, root length, fresh bulblet weight, fresh plantlet weight, and bulblet diameter (Canellas *et al.*, 2002; Zheng *et al.*, 2012). The fresh bulblet weight was estimated using an electronic balance (Mettler Toledo, PL202-L, Switzerland). The bulblet diameter was detected using an electronic digital vernier calliper (Links, China) and was only recorded at DAT60 and later time points. Ten independent plantlets were randomly selected for measurement of the above listed traits. Then the bulblets were thoroughly washed using distilled deionized (dd) water and frozen in liquid nitrogen (≥30 min), and stored at −75 °C for determination of major starch metabolic pathway traits.

2.3 Measurements of carbohydrate content and enzyme activity levels

First, the concentrations of major carbohydrates including starch, sucrose, and total soluble sucrose were measured by modified anthrone colourimetry, as described by McCready *et al.* (1950) and in our lab (Zheng *et al.*, 2012), and 0.5 g of tissue was used for each sample. The absorbance was determined at 620 nm.

For the assays of key enzymes in starch metabolism, 1 g frozen bulblet powder was taken and ground in liquid nitrogen with 5 ml enzyme extracting buffer (100 mmol/L Tricine-NaOH (pH 7.5), 8 mmol/L $MgCl_2$, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 50 mmol/L 2-mercaptoethanol, 12.5% (v/v) glycerol, and 1% (0.01 g/ml) insoluble polyvinylpyrrolidone-40). The homogenate was then centrifuged at 30 000*g* for 30 min, and the resulting supernatant was used as the preparation of ADPG pyrophosphorylase (AGPase, EC 2.7.7.27) and SSS (EC 2.4.1.21) while sediment was used for GBSS (EC 2.4.1.21). Reagents were purchased from Sigma-Aldrich (St. Louis, USA). All assays were carried out at 30 °C in the various reaction mixtures described as follows.

The AGPase assay was conducted in 100 mmol/L HEPES-NaOH (pH=7.4), 1.2 mol/L ADP-glucose, 3 mol/L inorganic pyrophosphoric acid (PPi), 5 mmol/L $MgCl_2$, 4 mol/L dithiothreitol (DTT), and enzyme preparation was in a reaction mixture of 130 μl. After 20 min, the reaction was terminated by heating the mixture in boiling water for 30 s. The resulting solution was transferred to a 1.5-ml Eppendorf tube and centrifuged at 10 000*g* for 10 min. A portion (100 μl) of the supernatant was taken and mixed with 5.2 μl colorimetric solution (5.76 mol/L nicotinamide adenine dinucleotide phosphate (NADP), 0.08 U phospho (P)-gucomutase, 0.07 U glucose-6-phosphate (G6P)-dehydrogenase). The activity was determined by measuring the increase in absorbance at 340 nm.

The SSS assay was conducted in 50 mmol/L HEPES-NaOH (pH=7.4), 1.6 mol/L ADPG, 0.7 mg amylopectin, and 15 mmol/L DTT, and enzyme preparation was in a reaction mixture of 56 μl. Twenty minutes later, the enzyme was inactivated by placing the mixture in a boiling-water bath for 30 s and was immediately cooled on ice for 20 s. Then the mixture was added to by 20 μl of a solution of 50 mmol/L HEPES-NaOH (pH=7.4), 4 mol/L phosphoenolpyruvate (PEP), 200 mol/L KCl, and 10 mmol/L $MgCl_2$, and pyruvate kinase (1.2 U), and incubated for 30 min at 30 °C in a water bath. The resulting solution was heated in boiling water for 30 s and then subjected to centrifugation at 10 000*g* for 10 min. The supernatant (60 μl) was mixed with a solution of 50 mmol/L HEPES-NaOH (pH=7.4), 10 mol/L glucose, 20 mmol/L $MgCl_2$, 2 mmol/L NADP, and pyruvate kinase (1.2 U), 1.4 U hexokinase, and 0.35 U G6P-dehydrogenase. The enzymatic activity was measured as the increase in absorbance at 340 nm.

The sediment was washed four times using 1 ml of the enzyme extracting buffer mentioned previously, and was detected using the same method as SSS. The whole enzyme estimation methods were slightly modified according to Nakamura *et al.* (1989).

The enzymes were compared on the basis of soluble protein content, and we used a modified Bradford method to determine the soluble protein (Bradford, 1976). Final concentrations in the solution were 0.01% (1 g/L) Coomassie Brilliant Blue G-250

(Ourchem®, 71011284, Sinopharm, China), 4.7% (0.047 g/ml) ethanol, and 8.5% (0.085 g/ml) phosphoric acid. Bulblet tissue (0.5 g) was ground with 5 ml distilled water and diluted to 10 ml. The homogenates were centrifuged at 10 000 r/min at 4 °C for 20 min. We used 20 μl supernate+80 μl distill water+5 ml solution, standing for 3 min. Absorbance at 595 nm was measured.

All the final samples (200 μl) were arranged in a 96-well enzyme label plate (Costar, 3590, USA) and spectrophotometric analysis was conducted on a Multilabel Reader (Perkin Elmer Corporation, Enspire™ 2300, USA). All treatment experiments consisted of three independent replicates.

2.4 Statistics

Data were statistically analysed using analysis of variance (ANOVA) by SPSS Version 13.0 software and tested for significant (P<0.05) treatment differences using the Duncan test. Bulblet fresh weight was also determined as a percentage of plantlet fresh weight (relative bulblet weight, RBW) (Gerrits and de Klerk, 1992). The radius (r) was derived from the diameter of the bulblets. Putting the value of r in the formula $4/3\pi r^3$, the volume of the bulblet was calculated (Thakur *et al.*, 2006). Bulblet swelling rate (mg/(plantlet·d))=(bulblet fresh weight$_{DAT((1+N)\times15)}$−bulblet fresh weight$_{DAT(N\times15)}$)/interval (15 d) (N=1, 2, 3, 4) (Wang *et al.*, 2008). Starch accumulation rate=(starch content$_{DAT((1+N)\times15)}$−starch content$_{DAT(N\times15)}$)/starch content$_{DAT(N\times15)}$ (N=1, 2, 3, 4) (Xiao *et al.*, 2013).

3 Results

3.1 Effects of non-HA and HA treatments on morphological traits of lily plantlets in vitro

The shoots were transplanted evenly with exogenous application of HA, and we considered seven critical morphological parameters and two derived indices to investigate the positive and/or negative effectiveness under HA treatments (Fig. 2). A quick response after HA treatments could be observed just at DAT15 (Table 1), and almost all the biomass indexes including plantlet height, number of leaves, number of roots, and root length increased significantly during the incipient bulblet stage. Thereafter, these traits were maintained at a relatively high level. In comparison, the controls changed stably (Table 1). It was noticeable to see that for plantlet height, MHA showed the lowest value (1.56 cm) at DAT15 while it increased dramatically to its highest at a later stage, e.g., 5.21 cm at DAT60. The number of leaves for LHA, MHA, and HHA was 7.7% to 38.5% higher than that of the control at DAT15, implying a possible stronger photosynthetic capacity. The number of leaves in MHA showed a similar trend as the plantlet height, viz., low at the beginning stage (2.8 at DAT15) but high at later stages (4.1 at DAT45). Taking both plantlet height and number of leaves together as major aboveground morphological traits, it can be concluded that for higher HA application, it caused relative vigorous aboveground growth at different phases (Table 1).

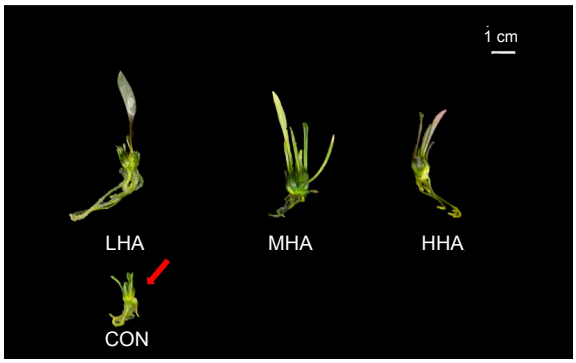

Fig. 2 Growth of control and HA application of in vitro *Lilium* Oriental Hybrids 'Sorbonne'
LHA: low concentration HA; MHA: moderate concentration HA; HHA: high concentration HA; CON: control. Bulblets were taken at DAT60. Arrow means the bulblet part

The number of roots during the former two stages for LHA was much greater than that of the control but not to a significant level (Table 1). Higher HA treatments (≥2.0 mg/L) only showed a slight superiority than the control until DAT45. Nevertheless, the average root length did not differ significantly between HA treatments and non-HA treatment, and the values for MHA and HHA were lower than that for the control. Despite the total fresh plantlet weight for HA ones all markedly exceeding that for the control, yet with respect to the bulblet weight there was only a smaller increase. This was also confirmed by relative bulblet weight. Accordingly, the RBW for LHA showed a range between 76.69% and 84.46%, whereas the average RBWs for MHA and

Table 1 Effects of humic acid (HA) treatments on morphological changes during *Lilium* Oriental Hybrids 'Sorbonne' plantlet development in vitro

Time	Plantlet height (cm)				Number of leaves			
	LHA	MHA	HHA	CON	LHA	MHA	HHA	CON
DAT15	2.07±0.15b	1.56±0.13b	3.14±0.24a	1.95±0.16b	3.10±0.57a	2.80±0.33a	3.60±0.22a	2.60±0.40a
DAT30	2.72±0.28a	2.94±0.36a	2.72±0.21a	2.13±0.19a	2.90±0.28a	3.50±0.75a	3.20±0.29a	2.50±0.40a
DAT45	2.86±0.42c	4.53±0.35a	3.70±0.50ab	2.00±0.34bc	2.60±0.31ab	4.10±0.48a	3.60±0.79a	1.40±0.40b
DAT60	3.52±0.40b	5.21±0.35a	4.58±0.42ab	3.60±0.58b	3.50±0.54a	3.60±0.34a	3.10±0.43ab	2.00±0.26b
DAT75	3.81±0.82e	–	3.63±0.22a	1.39±0.26b	2.80±0.44b	–	4.20±0.42a	1.40±0.26c

Time	Number of roots				Root length (cm)			
	LHA	MHA	HHA	CON	LHA	MHA	HHA	CON
DAT15	5.10±0.99a	3.50±0.45a	3.10±0.75a	3.20±0.65a	1.14±0.15a	0.98±0.11ab	0.57±0.12c	0.71±0.15bc
DAT30	7.40±0.92a	5.50±0.62a	5.70±0.58a	5.20±0.73a	4.35±0.28a	1.35±0.10b	1.34±0.12b	1.85±0.29b
DAT45	7.10±0.95a	7.80±0.84a	7.00±1.44a	5.50±1.09a	3.45±0.49a	1.63±0.16b	1.53±0.29b	2.70±0.58ab
DAT60	9.90±1.45a	5.80±0.80b	7.20±0.65ab	9.10±0.81a	4.18±0.59a	2.35±0.43b	2.01±0.29b	4.58±0.43a
DAT75	14.50±1.65a	–	9.00±0.87b	6.90±1.57b	5.75±0.34a	–	3.19±0.36b	3.18±0.78b

Time	Fresh bulblet weight (mg)				Fresh plantlet weight (mg)			
	LHA	MHA	HHA	CON	LHA	MHA	HHA	CON
DAT15	108.00±0.95a	93.00±1.40a	130.00±2.03a	109.00±1.39a	130.00±1.17b	111.00±1.64b	188.00±2.60a	142.00±1.46ab
DAT30	220.00±2.47a	215.00±2.88a	133.00±1.19b	159.00±1.75ab	283.00±3.14ab	315.00±5.50a	186.00±1.75b	193.00±1.86b
DAT45	193.00±2.16b	267.00±2.32a	174.00±2.43b	157.00±2.36b	254.00±2.68b	455.00±5.63a	304.00±5.21b	185.00±2.50b
DAT60	344.00±3.48a	294.00±2.75a	263.00±2.73a	256.00±2.48a	455.00±5.53a	454.00±4.39a	379.00±4.06a	331.00±3.75a
DAT75	468.00±6.29a	–	286.00±2.13b	160.00±3.77b	593.00±1.10a	–	379.00±2.98ab	170.00±4.09b

Time	Relative bulblet weight (%)[Z]				Bulblet diameter (mm)[Y]			
	LHA	MHA	HHA	CON	LHA	MHA	HHA	CON
DAT15	83.58±1.60ab	84.91±3.25a	68.38±3.09c	75.58±3.25bc				
DAT30	77.50±2.11ab	71.79±3.47b	72.16±2.49b	81.76±1.81a				
DAT45	76.69±3.46a	61.47±3.19b	61.53±5.10b	84.41±4.78a				
DAT60	78.22±3.71a	65.62±2.83b	69.99±3.25ab	78.37±2.81a	10.14±0.58a	9.23±0.50a	8.66±0.55a	8.81±0.44a
DAT75	84.46±3.55b	–	75.90±1.78c	96.19±2.02a	11.68±0.77a	–	9.47±0.59b	6.90±0.82c

Time	Bulblet size (mm^3)[YX]			
	LHA	MHA	HHA	CON
DAT15				
DAT30				
DAT45				
DAT60	593.00±101.41a	444.28±72.40a	377.41±69.23a	382.41±60.59a
DAT75	933.17±173.08a	–	493.70±98.01b	221.61±60.26c

DAT: days after transplanting; LHA: low concentration HA; MHA: moderate concentration HA; HHA: high concentration HA. Data are presented as means±standard error of the mean (SEM) with n=10. Mean separation within rows by Duncan's multiple range test at $P \leq 0.05$ (lowercase letter) for each trait. [Z] Relative bulblet weight (%)=fresh bulblet weight/fresh plantlet weight×100%. [Y] Data only recorded at DAT60 and after. [X] Bulblet size (mm^3)=$4/3\pi r^3$, r=1/2 bulblet diameter

HHA during the whole development stages were 70.95% and 69.59%, respectively. This phenomenon suggested that assimilates were utilized by up-ground vegetative growth especially for two higher HA treatments with the exception of LHA. LHA performed the best, resulting in an average bulblet yield of 468 mg, 10.14 mm for diameter, and 933 mm^3 for bulblet size at DAT75, 2.9-fold, 1.7-fold, and 4.2-fold, respectively, higher than those for the control. On the basis of bulblet swelling rate, it was high during P1, and a source-sink rebalance emerged at P2. Thereafter, it kept climbing to 8.27 mg/(plantlet·d) even at P4 (Fig. 3). At DAT60, the bulblet reached a mature status for HA treatments whereas some

indexes collapsed at DAT75 for CON, i.e., fresh bulblet weight experienced a sudden decrease to 160 mg (Table 1).

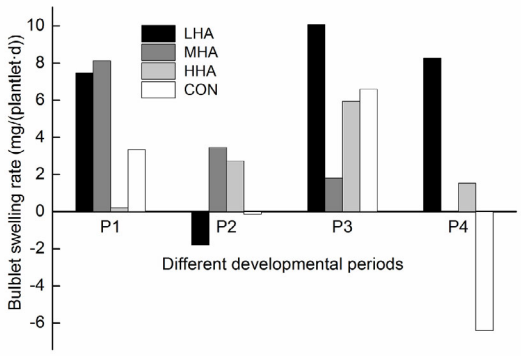

Fig. 3 Effects of humic acid (HA) on bulblet swelling rate during in vitro bulblet development of *Lilium* Oriental Hybrids 'Sorbonne'

P1, P2, P3, and P4 mean DAT15–DAT30, DAT30–DAT45, DAT45–DAT60, and DAT60–DAT75, respectively (DAT: days after transplanting)

3.2 Dose-response curves and time course changes of HA on non-structural carbohydrate accumulation in vitro

For the untreated control plants, the sucrose content changed steadily at around 8 mg/g FW, demonstrating that there is no evident substance transfer and utilization (Fig. 4a). When applied at moderate and higher concentrations (≥2 mg/L), sucrose exerted a similar gradual increase during the bulblet growing stage, and for HHA, it peaked at 14.61 mg/g FW, indicating efficient sucrose unloading and storage, particularly from aboveground. These were consistent with the observations in plantlet height and number of leaves, most of which were much higher than those having both LHA and CON treatments (Table 1). However, 0.2 mg/L HA had a relative low sucrose content compared with the other two HA treatments with two valley value at DAT15 (6.31 mg/g FW) and DAT60 (7.51 mg/g FW), which possibly meant that fast sucrose utilization and circulation was in progress. Total soluble sugar mainly consists of sucrose, fructose, maltose, glucose, etc. In the present study, it had almost the same trends as sucrose, only with greater values (Fig. 4b).

A promotive effect of HA application at early stage on starch enrichment was observed in all doses. From low to high HA concentrations, starch content was elevated by 15.3%, 31.0%, and 16.8%,

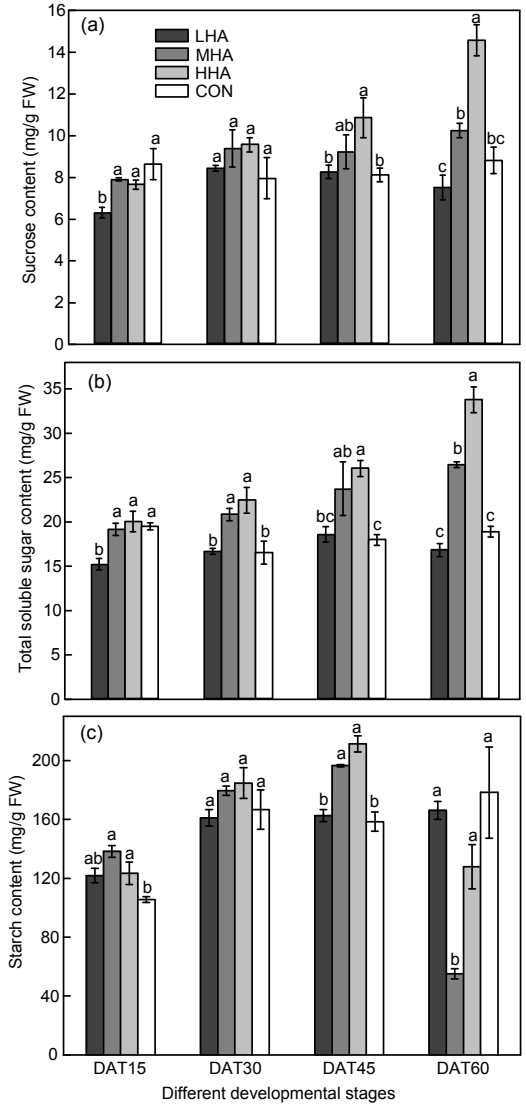

Fig. 4 Effects of humic acid (HA) on major non-structural carbohydrates during in vitro bulblet development of *Lilium* Oriental Hybrids 'Sorbonne'

The in vitro shoots were transplanted to medium containing basal MS plus 8 g/L agar and 70 g/L sucrose with different doses of HA, 0 (CON), 0.2 (LHA), 2.0 (MHA), and 20.0 (HHA) mg/L, respectively. For each treatment, 200 shoots were initially used. The bulblets were harvested at 9:00 a.m. on each sampling date. Data are means of three independent biological replicates. Push pin represents standard error (n=10). Means within each column denoted by the same lowercase letter do not significantly differ at P<0.05 according to the Duncan test. DAT: days after transplanting. (a) Sucrose content; (b) Total soluble sugar content; (c) Starch content

respectively. A continuous increase of starch accumulated until DAT45, suggesting that carbon fixation happened progressively. For instance, starch content

reached 211.42 mg/g FW for HHA at DAT45, which was 1.3-fold compared with the control. Accordingly, HA treatment could promote starch filling in a bulblet when carbon supply was sufficient in the medium in vitro. A slight increase in starch for LHA while a parallel collapse appeared for higher HA doses (Fig. 4c). The same phenomenon could be observed for LHA at DAT75. We found that starch for HHA (216.12 mg/g FW) began to accumulate again after a rebalance of source-sink.

By calculating the starch accumulation rate, we found that HA treatments strengthened starch synthesis and degradation compared with CON while LHA had a relatively delayed response to carbon starvation (Fig. 5).

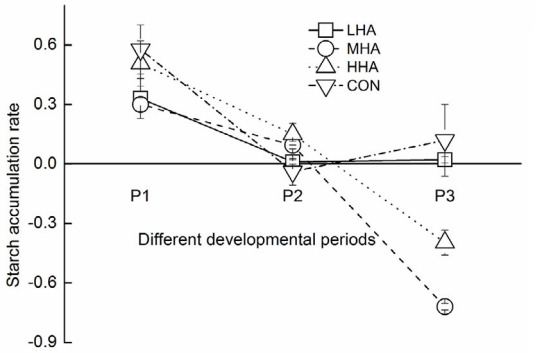

Fig. 5 Comparison of humic acid (HA) treatments to the starch accumulation rate of *Lilium* Oriental Hybrids 'Sorbonne' in vitro

Push pin represents standard error (n=10). P1 represents DAT15–DAT30; P2 represents DAT30–DAT45; P3 represents DAT45–DAT60

3.3 Dynamic changes of starch metabolism-related enzymes in lily bulblets in vitro

At low concentrations (0.2 mg/L), HA treatment could enhance all the three enzyme activities very significantly to an extraordinarily high level of 29.4, 7.0, and 28.8 nmol/(mg protein·min) for AGPase, GBSS, and SSS at DAT15, respectively, which were higher than those in MHA by 150.54%, 112.40%, and 120.63%, respectively. AGPase thus persisted at 29.0 nmol/(mg protein·min) at DAT30 while both GBSS and SSS continually enhanced by 2.3 and 1.3 times, respectively. Thereafter, all the three enzymes showed a gradual decline until DAT60 (Fig. 6).

A similar tendency in enzyme activity was observed again for higher HA application (2.0 and 20.0 mg/L). An inhibition emerged compared with

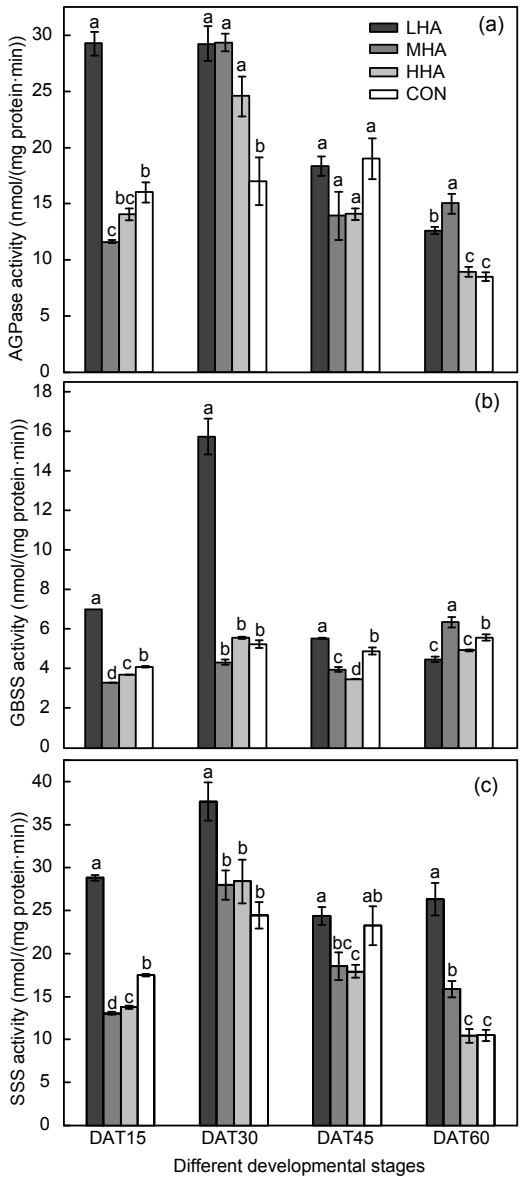

Fig. 6 The dose- and time-course response of humic acid (HA) on starch synthesizing enzyme activities during in vitro bulblet development of *Lilium* Oriental Hybrids 'Sorbonne'

The in vitro shoots were transplanted to a medium containing basal MS plus 8 g/L agar and 70 g/L sucrose with different doses of HA, 0 (CON), 0.2 (LHA), 2.0 (MHA), 20.0 (HHA) mg/L, respectively. For each treatment, 200 shoots were initially used. The bulblets were harvested at 9:00 a.m. on each sampling date. Data are means of three independent biological replicates. Push pin represents standard error (n=10). Means within each column denoted by the same lowercase letter do not significantly differ at P<0.05 according to the Duncan test. DAT: days After Transplanting. (a) ADPG pyrophosphorylase (AGPase) activity; (b) Granule-bound starch synthase (GBSS) activity; (c) Soluble starch synthase (SSS) activity

untreated control plants at DAT15, as opposed to the elevation in LHA. However, AGPase and SSS activities were rapidly elevated at DAT30, which apparently implied an accelerated starch metabolic pathway. Although we cannot precisely explain the reason for the lower starch enzyme activity with respect to the higher amount of starch at DAT15 for these two treatments, we assume that it might possibly be attributed to transitory starch transportation from leaf to bulblet and low starch break down enzymes. By contrast, GBSS changed little, and therefore, amylopectin may account for the greater proportion in higher HA treatment plants. For control plants, three enzymes changed relatively more stably, especially for AGPase and GBSS. Taking GBSS as an example, the values just held at around 4.09–5.57 nmol/(mg protein·min) (Fig. 6b).

4 Discussion

Reproduction of lily bulbs can be achieved through various approaches including tissue culture. The formation and development of bulblets are crucial in the life cycle (Li *et al.*, 2014). Despite lots of literature on micropropagation of *Lilium* (Joshi and Dhar, 2009; Saadon and Zaccai, 2013), the physiological and biochemical mechanisms governing bulblet development remain unclear. Much evidence has shown that HA favors plant growth in many ways but is limitedly used in bulbous crops including in vitro. Lower molecular size fraction of HA, e.g. fulvic acid, can easily reach the plasmalemma of higher cells and therefore is the major candidate for determining the positive effects of HA on plant growth (Nardi *et al.*, 2002). In our experiment, HA containing more than 90% fulvic acid was chosen, and the beneficial aspects were substantiated by the promotive effect especially at lower concentration.

We found that HA accelerated the aboveground leaves' growth to a different extent in lily (Table 1), which was in accordance with the previous report of HA treatments in cucumber (Mora *et al.*, 2010). However, Adani *et al.* (1998) showed contradictory results in tomato plants using hydroponics culture. CP-A (commercially-available HA products prepared from peat) stimulated root growth only while having no impact on shoots at 20.0 mg/L. The HA effect on shoot growth might be closely related to several factors, e.g., species sensitivity, pH condition (de Kreij and Başar, 1995), HA origins (Adani *et al.*, 1998), and medium containing N or not (Malik and Azam, 1985). Research has also shown that HA increased chlorophyll content which can accordingly strengthen photosynthesis (Xu, 1986; Liu *et al.*, 1998). Sufficient mature functional leaves promoted photosynthesis (Zheng *et al.*, 2012) during the day, and the fixed carbon, like sucrose, is transported from the photosynthetic apparatus to the non-photosynthetic organs during the night (Bahaji *et al.*, 2014). That is, more vigorous leaves gave rise to sucrose accumulation.

Previous reports have demonstrated that HA can facilitate the plant rhizosphere environment and biological characteristics (Canellas *et al.*, 2002). HA was applied to improve the post-rooting stage in American chestnut shoots with difficulty in rooting as tree species (Oakes *et al.*, 2016). We also reported that 500 mg/L HA combined with 7 mmol/L Ca favored root growth in the Oriental lily 'Sorbonne' (Chang *et al.*, 2012). For the in vitro lily, we observed that low HA produced more roots than CON, e.g., to 14.5 at DAT75 for LHA (Table 1). However, higher HA concentrations (≥2.0 mg/L) showed an inhibitive effect on root length. In comparison, 0.2 mg/L HA performed best both in root number and root length (Table 1). The huge difference in effective concentration between field and tissue culture might be due to two reasons: (1) iron-reducing, fermenting bacteria etc. in the soil may lead to HA reduction (Benz *et al.*, 1998); (2) plantlets in vitro absorb HAs in the medium more directly. As such, we concluded that it is necessary to take the dose effect into consideration when using HA and that the in vitro condition is more sensitive. Too high HA may cause a repression effect, which was in line with results in tomato and cucumber (Atiyeh *et al.*, 2002). Thus, in terms of shoot and roots traits, the effects were the reverse in LHA and higher HA treatments (Table 1). It was reported that the mechanism responsible for shoot growth of HA might be ascribed to the enhancement in root H^+-ATPase activity and the increase in nitrate shoot concentration, which in turn causes root-shoot distribution (Mora *et al.*, 2010). Tao *et al.* (1987) used HAs by different application methods (spray, drench, and soaking) and various concentrations (50–300 ppm (1 ppm=1 mg/L)). They found that 100 ppm had the most effective result and that increased potato yield per mu (1 mu=1/15 ha) by 11.4% to 33.5%

compared with the control trial. The larger tuber held a much greater proportion in total tuber number and the small tuber was only 9.55%–22.1% that of control. The weight of extra-large tubers increased in the fulvic acid treated potato (Suh *et al.*, 2014). The bulblet size (weight and volume) was smaller for higher HA doses (≥2.0 mg/L) than for LHA. In terms of morphogenic traits only, two factors might be involved. Firstly, less robust roots would affect the carbon and nutrition absorption rate from the culture medium; secondly, excessive aboveground growth entailed extra energy and nutrition consumption. Although more leaves gained more photosynthesis, a relatively low average relative bulblet weight during the whole development process for MHA (70.95%) and HHA (69.59%) verified assimilates allocation between shoot (leaves) and root (bulblet) (Gerrits and de Klerk, 1992).

Intriguingly, a sharp decrease was found in fresh bulblet weight for CON at DAT75. In *Lilium rubellum* in vitro culture, three sugars (sucrose, glucose, and fructose) were detected in the autoclaved MS medium which only contained sucrose. Sugars in the 150 mmol/L (about 51.34 g/L) sucrose medium were nearly depleted by Week 8, whereas those in the 250 mmol/L sucrose medium persisted for 12 weeks (Niimi *et al.*, 2000). Similar sucrose depletion was observed during *Lilium japonicum* bulblet swelling (Yamagishi, 1998). Thus, it was certain that 70 g/L had already been used up in the medium (carbon starvation) whilst the limited number of leaves (2) was not enough to produce carbon to support further growth both in the aboveground part and the bulblet. Carbohydrates are hypothesized to modulate expression of lily developmental genes, a phenomenon known as sugar sensing (Koch, 1996). Sucrose is required as the carbon source (Takayama and Misawa, 1979) and serves as the primary transport carbohydrate in *Lilium*. In other words, sucrose is the end product of photosynthesis in leaves for translocation from the source (leaf) to heterotrophic sink (bulblet) through the phloem (Ruan, 2014) and can be utilized directly for plant growth. Accumulation of total soluble sugars was observed after exogenous HA application and sucrose was the dominant one (Figs. 4a and 4b). This was similar to the observation in the edible sweetpotato which had significantly increased soluble sugar content in storage organs (Liu *et al.*, 2011). Use of vermicompost in garlic also corresponded to an increase in the total soluble carbohydrates (Argüello *et al.*, 2006). In addition, soluble carbohydrates accumulated more with increase in HA doses in the present study (Fig. 4). Sucrose is not only the carbon source but also important for molecular signals (Koch, 2004). When sucrose is imported into the sink organ, it will be degraded into hexose or hexose phosphate to take part in cell metabolism or it will result in "sucrose accumulation". The degradation direction of sucrose determines the fate of assimilate utilization (Sonnewald and Uwe, 2012). Therefore, the relatively low sucrose content in LHA treatment was noticeable with two valley values at DAT15 and DAT60 (Fig. 4b). These were well correlated with the morphological traits including fresh bulblet weight since two vegetative growth peaks emerged at DAT15 and DAT60 for LHA, implying that sucrose was used not only for energy metabolism but also for storage metabolism during these two development stages (Sonnewald and Uwe, 2012).

Starch biosynthetic and degradative genes in higher plants show high conservation in their biological roles (Tetlow *et al.*, 2004). Generally, three enzymes, including AGPase, GBSS, and SSS, were directly involved in starch synthesis (Smith *et al.*, 1997). HA application enhanced starch accumulation with dose increase and this was confirmed by high starch synthetic enzymes activity. LHA exerted the highest AGPase, GBSS, and SSS activities (Fig. 6a). The elevated starch enzyme activity was in synchrony with the lowest sucrose content in LHA treatment which was presumably due to high sucrose use efficiency. Similar findings were obtained in PGRs' foliar spray treatment in lily bulbs during the full- and post-blossoming periods (Zheng *et al.*, 2012). Also, AGPase reached a very high level at DAT15 compared with GBSS and SSS (Fig. 6a), which confirmed the precursor and substrate role of ADPG to fully support the further amylose and amylopectin synthesis (Cheng *et al.*, 2015). Notably, despite high starch synthetic enzymes, starch content was lowest among HA treatments for LHA (Fig. 4c). It was previously demonstrated that HA application in sweetpotato significantly elevated α-amylase and β-amylase activities by 11.33% and 15.70%, respectively (Liu *et al.*, 2011). Therefore, we proposed that for LHA both starch synthesis and degradation directions were extremely active. Also, at DAT60, an earlier decrease in starch was observed for MHA and HHA while

LHA delayed the carbon famine (Fig. 4b). As mentioned above, carbon in the medium was totally depleted around DAT60. Hence, it was a necessity to hydrolyse the starch in the bulblets into soluble sugars supporting plantlets growth at DAT60, which was in line with the consecutive increased sucrose content at the very same time point (Fig. 4a). Thus, it was a source-sink rebalance and conversion process.

5 Conclusions

Bulbous plants greatly contribute to the world flower industry while understanding of bulb development is very limited and this produces a bottleneck in the improvement of propagation and the breeding programme of new bulbous cultivars. To interpret the mechanisms of bulb development, we examined bulbs in vitro and HA was first applied. A range of morphology indices were determined which showed either root or leaf growth was elevated compared with the control treatment depending on the HA concentrations used. A significant promotion in bulblet diameter and weight was obtained with no deformity in LHA. We investigated the major compounds and enzymes involved in the starch synthesis pathway combined with HA dose effects. The lowest sucrose contents whilst highest AGPase, GBSS, and SSS activities for LHA were all observed as early as DAT15, implying the high efficiency in carbon utilization and source-sink conversion. Higher concentrations of HA (2.0 and 20.0 mg/L) facilitated both sucrose and starch accumulation during the growing stage as compared with the control plants. LHA was the better choice. The average fresh bulblet weight and diameter could reach 468 mg and 11.68 mm, respectively. HA treatments are promising for use as an organic substance in bulblet production as well as being without residue to the soil or the environment. However, the detailed mechanisms need further clarification by taking both amylase and photosynthesis traits into consideration.

Compliance with ethics guidelines

Yun WU, Yi-ping XIA, Jia-ping ZHANG, Fang DU, Lin ZHANG, Yi-di MA, and Hong ZHOU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 Classic interpretation of source-sink-conversion pathway in in vitro bulblet

中文概要

题　目：低浓度腐殖酸处理通过促进根系发育及碳水化合物代谢促进百合离体小鳞茎膨大

目　的：小鳞茎发育问题研究是缩短百合育种周期、加快百合种球产业化生产的关键，而碳水化合物代谢则是影响小鳞茎发育的重要因子。离体快繁技术因更高效的繁殖速率及易控的环境条件，是鳞茎发育问题研究的替代途径，但目前有关离体条件下鳞茎发育机制研究鲜有报道。本文采用腐殖酸处理东方百合'索邦'，研究其对于鳞茎膨大的影响及碳水化合物代谢调控路径。

创新点：本研究首次采用安全无残留型植物生长调节物质腐殖酸处理，并探讨了其对百合离体鳞茎发育的有效影响及内在的碳水化合物代谢生理生化机制。

方　法：以构建的离体模式体系下形成的百合单芽，接种至含不同浓度腐殖酸（0、0.2、2.0 和 20.0 mg/L）的培养基上，每隔 15 天取样一次，测定株高等 7 个形态指标；同时，取鳞茎测定主要非结构性碳水化合物（蔗糖、可溶性糖、淀粉）含量及关键淀粉合成酶（AGPase、SSS 和 GBSS）活性。

结　论：随腐殖酸处理浓度升高，相对鳞茎重量下降，从而打破库–源平衡，低浓度腐殖酸（0.2 mg/L）处理效果最佳，鳞茎重量为 468 mg（为对照的 2.9 倍），鳞茎直径达 11.68 mm。具体来说，低浓度腐殖酸处理可促进根系发育，并在发育早期大幅促进淀粉合成相关酶活性，并通过加速蔗糖/淀粉利用及转换延缓了碳饥饿出现时间。

关键词：小鳞茎发育；腐殖酸；淀粉代谢；库–源转换；东方百合'索邦'