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Original Article

Investigation of agrobiological properties of *ctb* (cholera toxin B subunit) transgenic tomato under *in vivo* condition

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Abstract

The study aimed to evaluate some agrobiological properties of *ctb* transgenic tomatoes under *in vivo* condition through physiological and biochemical characteristics relate to fruit yield and quality. Agrobiological parameters are determined by weighing, measuring and counting. Photosynthetic rate was determined via uptake carbon dioxide, the spectrophotometric method used to measure chlorophyll and total carotenoid content, vitamin C was determined using the iodine titration method, reducing sugar was determined by dinitrosalicylic acid, degree Brix was measured using an ATAGO N1 refractometer and total acidity in fruit juice was determined by neutralization method. Study results showed that final harvesting time for all tomatoes were 150 days including transgenic plants and control. Plant height (cm) ranged from 80.3 to 83.6, number of compound leaves from 17.6 to 22, and number of inflorescences from 7.3 to 9.3. The chlorophyll content (mg/g) and the photosynthetic rate (µM $CO_2/m^2/s$) peaked at young fruit stage in both transgenic plants and control with values from 0.48 to 0.62 and from 9.08 to 16.77, respectively. The yield, yield components and fruit shape of transgenic plants and control were also similar. Number of fruits ranged from 14.6 to 23, fruit weight (g) ranged from 61.5 to 69.3, and individual yield (kg) varied from 0.99 to 1.53. The main biochemical characteristics of transgenic plants and control were not different, dry matter (%) accounts for 5.45-5.91, reducing sugar (%) of 1.87-2.22, vitamin C (mg/100 g) of 44.01-46.13, acidity (%) of 0.62-0.89, Brix (%) of 5.23-6.01 and carotene (mg/100 g) of 3.01-3.84. In conclusion, six *ctb* transgenic tomato individuals were able to grow normally under *in vivo* conditions similar to non-transgenic control plants. Agrobiological properties between transgenic plants and control were insignificantly different with $p>0.05$.

Keywords: Cholera toxin B subunit (CTB), *In vivo ctb* transgenic tomato, *Lycopersicon esculentum*, *Vibrio cholerae*

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Introduction

Tomato (*Lycopersicon esculentum* L.) is a very nutritious fruit vegetable, have good flavors and attractive colors, so they are often used in food processing. This is an important agricultural crop with a global productivity in 2007 of about 120 million tons (Passam et al., 2007) and about 181 million tons in 2017 as reported by FAO (2019, with date reference on May 1st, 2019). Tomatoes contain ingredients that have high nutritional values, such as potassium, folate, vitamin C, carotenoids (lycopene, β-carotene, γ-carotene, phytoene and phytosterols), flavonoids, vitamin E and some water-soluble vitamins (Beecher, 1998).

In past years, some studies used tomato as a plant host for expression of antigen proteins to produce vaccines for human and animal such as proteins that cause plague and pneumonia (Alvarez et al., 2006), hepatitis B surface antigen (HbsAg) (Srinivas et al., 2008), human immunodeficiency virus (HIV) antigen, protein N of rabies virus (Perea-Arango et al., 2008), cholera toxin B subunit of *Vibrio cholerae* (CTB) (Loc et al., 2011), antigenic polypeptide containing epitopes of the diphtheria, pertussis and tetanus exotoxins (Soria-Guerra et al., 2007), malaria antigen (PfCP-2.9) (Kantor et al., 2013), *E. coli* heat-labile enterotoxin B subunit (LTB) (Loc et al., 2014). However, there has been little research on the agronomic characteristics of transgenic tomato plants planted natural condition (Prematilake et al., 2002; Shah et al., 2015).

In some species, transgenic plants were evaluated for a number of field trials such as *Arabidopsis thaliana* and tobacco (Lieman-Hurwitz et al., 2003), rice (Chen and Xu, 2007), tobacco and cotton (Rawat et al., 2011), tobacco (Wang et al., 2012), cucumber (Kiełkiewicz et al., 2012), pea (Reinecke et al., 2013), watercress (Loc et al., 2015). This work, therefore, aims to investigate the physiological and biochemical characteristics related to yield and fruit quality of *ctb* transgenic tomatoes, a gene encoding the CTB antigen which is a potential candidate for cholera vaccine, to evaluate their growth and development under *in vivo* condition.

Material and Methods

Plant materials

Six *ctb* transgenic tomato (*L. esculentum* L. cv. 311) individuals through *Agrobacterium tumefaciens*-mediated transformation from a previous study (Loc et al., 2011) were used to evaluate their growth and development under *in vivo* condition. 180 transgenic plants (6 types of individuals×30 plants of each type) grow normally under *in vitro* conditions with healthy roots have been planted in pots containing the ratio of 2 part of sandy soils, 1 part of coconut fiber and 2 parts of bio-compost, and placed in a net house for study. The distance from one pot to another was 60 cm. The farming techniques, including the uniformity of biological materials, were applied evenly for all. Thirty *in vitro* non-transgenic tomato (*L. esculentum* L. cv. 311) plants were used as the control.

Tomato cultivar 311 was supplied by Dai Dia Co. Ltd. (Vietnam). This is a heat-tolerant tomato cultivar that can be grown year round. The fruits are round and flattened, weight of 90-100 g/fruit, firm and thick flesh. Seedlings 20-25 days old can be planted. Time to start harvesting is 60 days after planting.

Physiological characteristics

The growth and development of tomato plants were split into five stages including branching, flowering, fruiting, first and final harvest for evaluation. In addition, data on plant height, number of compound leaves and inflorescences were also collected.

Total chlorophyll content of leaf was determined using the spectrophotometric method as described by Li et al. (2018) with a slight modification. In brief, 0.1 g fresh leaves were extracted with 95% ethanol, the filtrate was then used to measured chlorophyll at wavelengths of 649 and 665 nm. The contents of chlorophyll a and chlorophyll b were calculated as following equations (Lichtenthaler and Buschmann, 2001):

Chlorophyll a (µg/mL) = $13.36\times A_{665} - 5.19\times A_{649}$

Chlorophyll b (µg/mL) = $27.43\times A_{649} - 8.12\times A_{665}$

Where: A_{649} and A_{665} are absorbances of extract at 649 and 665 nm. Chlorophyll content (µg/mL) was then converted to mg/g leaf.

Photosynthetic rate (PR) was determined via uptake carbon dioxide (Field et al., 1989) using LI-6800 portable photosynthesis system, where the leaf is enclosed in a small transparent chamber. The rate of carbon dioxide fixed by the leaf is determined by measuring the change in the carbon dioxide concentration of the air flowing across the chamber.

Biochemical characteristics

Tomato fruits were used to analyze some biochemical characteristics related to their quality. Total

carotenoid content was determined by the spectrophotometric method. Leaf extract was prepared as for chlorophyll and carotenoid calculated as follows (Lichtenthaler and Buschmann, 2001):

Carotenoid (µg/mL) = $(1000\times A_{470} - 2.13\times C_a - 97.64\times C_b)/209$

Where: A_{470} is absorbance of leaf extract at 470 nm. C_a and C_b are the contents of chlorophyll a and chlorophyll b which determined as described above. Carotenoid content (µg/mL) was then converted to mg/100 g leaf.

Vitamin C content was determined using the iodine titration method (Njoku et al., 2011) with a slight modification. Twenty milliliters of tomato extract was added 25 mL of distilled water and 1 mL of 2% starch indicator solution. The titration was then carried out with standard iodine solution (for 100 mL: 1 g KI, 53.6 mg KIO_3 and 6 mL 3M H_2SO_4). The endpoint of the titration occurred when permanent dark blue-black color was obtained due to the starch-iodine complex. The ascorbic acid was used as the standard and the vitamin C content in the extract was calculated as follows:

Vitamin C (%) = $100(VI_A/VI_E)$

Where: VI_A is the volume of standard iodine solution that reacts with ascorbic acid. VI_E is the volume of standard iodine solution that reacts with vitamin C in the extract.

Reducing sugar was determined by dinitrosalicylic acid method (De Toledo et al., 2012) with a slight modification. 5 g tomato fruit were ground in 5 mL of 0.2 M phosphate buffer (pH 7), the homogenate was then centrifuged at 12000 rpm for 30 min, and the supernatant was used for detection of reducing sugar. 0.2 mL of the supernatant was mixed with 1.5 mL of 3,5-dinitrosalicylic acid and 1.8 mL of double-distilled water; the mixture was heated at 100°C for 5 min, cooled at room temperature and then added double-distilled water to a final volume of 25 mL. Reducing sugars were determined spectrophotometrically at 540 nm and the results were expressed as percentage of fresh weight with glucose was used as a standard.

Degree Brix was measured using an ATAGO N1 refractometer (Japan).

Total acidity in fruit juice was determined by neutralization method (Sadler and Murphy 2010). In brief, 10 g tomato fruit were extracted with double-distilled water at 80ºC for 15 min, then filtered and brought to a volume of 250 mL, followed by cooling at room temperature. 25 mL of filtrate was transferred to a new conical flask, added 3 drops of 0.1% phenolphthalein, then titrated with 0.1 N NaOH until a light pink color was obtained. Total acidity (%) of the extract was calculated as followed:

Acidity (%) = $100(V\times K)/V_1$

Where: V is the volume (mL) of 0.1 N NaOH, V_1 is the volume (mL) of the extract, and K is the adjustment coefficient (0.0064 for citric acid).

Statistical analysis

The experiments were carried out with at least ten replicates for each type of sample (n = 10×7 sample types, 6 transgenic individuals and 1 control) and each experiment was repeated 3 times. The data were statistically treated by ANOVA (Duncan's test at 0.05) using SPSS software and expressed as the mean of repeats.

Results

Physiological characteristics

Data from Table 1 show that the growth and development stages of six *ctb* transgenic and control plants are insignificantly different.

Table-1. Stages of growth and development (days) of *ctb* transgenic tomato plants compared with control (non-transgenic tomato plant)

Transgenic plants	Branching	Flowering	Fruiting	1st harvesting	Final harvesting
1	12	31	36	66	150
2	14	32	38	68	150
3	12	32	40	69	150
4	16	31	38	68	150
5	14	33	40	70	150
6	12	33	39	68	150
Control	12	32	38	67	150

Their final harvest time is 150 days while the stages of branching, flowering, fruiting and first-time harvesting only differ from 1 to 2 days. Study on the growth and development characteristics of transgenic and control tomatoes at flowering time including plant height, number of compound leaves and number of inflorescences also found no significant differences. Plant height ranges from 80.3 to 83.6 cm, compound leaf number was from 17.6 to 22, and inflorescence number was from 7.3 to 9.3 (Table 2).

Table-2. Characteristics of growth and development of *ctb* transgenic tomato plants compared with control (non-transgenic tomato plant)

Transgenic plants	Plant height (cm)	No of compound leaves	No of inflorescences
1	83.6^{a}	19.0^{b}	8.0^{a}
2	81.0^{a}	18.0^{b}	8.0^{a}
3	80.3^{a}	19.6^{ab}	8.3^{a}
4	80.3^{a}	17.6^{b}	7.3^{a}
5	82.0^{a}	18.6^{b}	7.6^{a}
6	83.3^{a}	22.0^{a}	9.3^{a}
Control	81.6^{a}	18.6^{b}	8.6^{a}
$LSD_{0.05}$	4.05	2.56	2.93

Significant differences between mean values are represented by different letters in a column at confidence level of 0.05 of Duncan's test.

Table-3. Chlorophyll content (mg/g) of *ctb* transgenic tomato plants compared with control (non-transgenic tomato plant)

Stages	Transgenic plants						Control	$LSD_{0.05}$
	1	2	3	4	5	6		
Branching	0.23^{a}	0.26^{a}	0.22^{a}	0.20^{a}	0.23^{a}	0.27^{a}	0.24^{a}	0.09
Flowering	0.49^{bc}	0.51^{b}	0.45^{de}	0.44^{e}	0.47^{cd}	0.53^{a}	0.49^{bc}	0.02
Young fruit	0.50^{b}	0.60^{a}	0.49^{b}	0.48^{b}	0.52^{b}	0.62^{a}	0.52^{b}	0.05
Green ripe fruit	0.46^{b}	0.55^{a}	0.46^{b}	0.45^{b}	0.49^{b}	0.57^{a}	0.48^{b}	0.04
Red ripe fruit	0.26^{a}	0.24^{ab}	0.22^{b}	0.23^{ab}	0.22^{b}	0.21^{b}	0.22^{b}	0.03

Significant differences between mean values are represented by different letters in a row at confidence level of 0.05 of Duncan's test.

The chlorophyll content (mg/g) of six *ctb* transgenic plants range from 0.20 to 0.27 in branching stage (control: 0.24), 0.44 to 0.53 in flowering stage (control: 0.49), 0.48 to 0.62 in young fruit stage (control: 0.52), 0.45 to 0.57 in green ripe fruit stage (control: 0.48), and 0.21 to 0.26 in red ripe fruit stage (control: 0.22) (Table 3). Unlike chlorophyll content, the PR (μM $CO_2/m^2/s$) of *ctb* transgenic plants was relatively different, they range from 7.94 to 14.89 in branching stage (control: 12.76), 8.94 to 16.38 in flowering stage (control: 15.01), and 9.08 to 16.77 in young fruit stage (control: 15.42), where transgenic plants #3 and #4 had the lowest PR. During the two ripening stages of tomato, the PR of the transgenic and control plants was not significantly different (Table 4).

Table-4. Photosynthetic rate (μM $CO_2/m^2/s$) of *ctb* transgenic tomato plants compared with control (non-transgenic tomato plant)

Stages	Transgenic plants						Control	$LSD_{0.05}$
	1	2	3	4	5	6		
Branching	10.67^{bc}	14.64^{a}	8.69^{cd}	7.94^{d}	13.16^{ab}	14.89^{a}	12.76^{ab}	2.15
Flowering	12.76^{b}	15.14^{ab}	9.18^{c}	8.94^{c}	14.52^{ab}	16.38^{a}	15.01^{ab}	1.98
Young fruit	13. 40^{a}	16.38^{a}	9.68^{b}	9.08^{b}	16.08^{a}	16.77^{a}	15.42^{a}	3.65
Green ripe fruit	6.70^{a}	7.33^{a}	5.66^{a}	5.58^{a}	6.70^{a}	7.63^{a}	6.52^{a}	2.40
Red ripe fruits	4.10^{a}	3.72^{a}	3.72^{a}	3.91^{a}	3.54^{a}	4.06^{a}	4.84^{a}	1.67

Significant differences between mean values are represented by different letters in a row at confidence level of 0.05 of Duncan's test.

Data from Table 5 and Fig. 1 show that the yield, yield components and fruit shape of *ctb* transgenic and control tomato plants are similar, except fruit number of transgenic plants #3 and #4 are relatively low. The fruit number of six transgenic plants range from 14.6 to 23 (control: 20.6), their fruit weight range from 61.5 to 69.3 g (control: 63.4 g), and individual yield varies from 0.99 to 1.53 kg (control: 1.31 kg).

Table-5. Yield and yield components of *ctb* transgenic tomato plants compared with control (non-transgenic tomato plant)

Transgenic plants	No of fruits	Fruit weight (g)	Individual yield (kg)
1	19.6^{ab}	61.9^{ab}	1.21^{a}
2	21.3^{ab}	66.6^{a}	1.42^{a}
3	14.6^{b}	68.1^{a}	0.99^{ab}
4	15.6^{b}	69.3^{a}	1.08^{ab}
5	20.3^{ab}	61.5^{ab}	1.25^{a}
6	23.0^{a}	66.4^{a}	1.53^{a}
Control	20.6^{ab}	63.4^{a}	1.31^{a}
$LSD_{0.05}$	5.04	7.92	0.52

Significant differences between mean values are represented by different letters in a column at confidence level of 0.05 of Duncan's test.

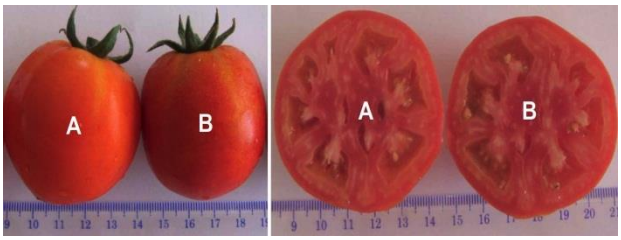

Figure-1. Tomato fruit shape is flattened of control plant (A) and *ctb* transgenic plant (B).

Biochemical characteristics

Data from Table 6 show that almost the analyzed biochemical characteristics of six transgenic and control tomato plants are not different. Dry matter (%) is from 5.45 to 5.91 (control: 5.62), reducing sugar (%) is from 1.87 to 2.22 (control: 2.04), vitamin C (mg/100 g) is from 44.32 to 46.13 (control: 44.01), acidity (%) is from 0.62 to 0.89 (control: 0.82), Brix (%) is from 5.23 to 6.01 (control: 5.89), and finally carotenoid (mg/100 g) from 3.01 to 3.84 (control: 3.66). CTB protein content of transgenic tomatoes reached a value of about 0.9% of total soluble protein as reported in a previous study (Loc et al., 2011).

Table-6. Tomato fruit quality of *ctb* transgenic plants compared with control (non-transgenic tomato plant)

Transgenic plants	Dry matter (%)	Reducing sugars (%)	Vitamin C (mg/100 g)	Acidity (%)	Brix (%)	Carotenoid (mg/100 g)
1	5.91^{a}	2.13^{a}	45.02^{b}	0.62^{ab}	5.74^{a}	3.62^{a}
2	5.48^{a}	1.98^{a}	46.13^{a}	0.78^{a}	5.67^{a}	3.60^{a}
3	5.45^{a}	2.22^{a}	44.57^{b}	0.81^{a}	5.23^{a}	3.01^{a}
4	5.78^{a}	2.09^{a}	44.55^{b}	0.89^{a}	6.01^{a}	3.84^{a}
5	5.82^{a}	1.87^{ab}	45.77^{a}	0.69^{a}	5.55^{a}	3.43^{a}
6	5.45^{a}	1.95^{a}	44.32^{b}	0.71^{a}	5.72^{a}	3.55^{a}
Control	5.62^{a}	2.04^{a}	44.01^{b}	0.82^{a}	5.98^{a}	3.66^{a}
$LSD_{0.05}$	0.61	0.54	0.88	0.30	1.12	1.01

Significant differences between mean values are represented by different letters in a column at confidence level of 0.05 of Duncan's test.

Discussion

In a previous study, we found that there were no remarkable differences about root number and length or there were only slight differences about plant height and leaf number between *ltb* transgenic watercress (*Nasturtium officinale*) and control plant. In the whole, their physiological characteristics were also insignificantly different (Loc et al., 2015). Before that, Wei et al. (2004) showed the PR of *NADP-ME* transgenic rice line and control rice plant were similar. Thiruveedhi (2006) indicated that chlorophyll and carotene content in *AtGDH* and *GTA* transgenic tobacco lines and control tobacco were also equivalent. According to Prematilake et al. (2002), the number of chloroplasts or stomatal guard cell pairs in *npt*II transgenic tomato lines and control plants was not different. Several other reports showed no differences about morphology, growth rate and flowering time between transgenic and control plant such as transgenic cucumber resistant to *Botrytis cinerea* (Koga-Ban et al., 2004) or *nptII* transgenic tomato (Prematilake et al., 2002).

Investigation of some growth and morphological characteristics of *Gus* and *nptII* transgenic *Solanum dulcamara* plants showed that their plant height, length of stem nodal segment, length and width of leaf blade were insignificant different in comparison with control (Curtis et al., 2000). However, Shah et al. (2015) found that when treated with cold stress, the conductivity of stomata, the rate of evaporation and the relative water content in *DREB1A* transgenic tomatoes were significantly higher than the control plants.

Study of Baxter et al. (2015) showed that *PvMYB4* transgenic switchgrass (*Panicum virgatum*) had important gains in both biofuel (more than 32%) and biomass (more than 63%) at the end of the second growing season compared to control plant. However, we did not find significant differences in biochemical characteristics such as contents of pigment, cellulose, vitamin C, calcium and potassium of *ltb* transgenic watercress compared to the control when were planted under natural condition (Loc et al., 2015). Xu et al. (2018) also obtained similar results from transgenic rice expressed Cry1Ab/Vip3A fused protein for insect resistance. Their agronomic characteristics such as plant height, panicles per plant, grains per panicle, weight of 100 grains and seed set rate showed insignificant differences in comparison with control rice plant.

Conclusion

From this investigation, we find that six *ctb* transgenic tomato individuals with the normal phenotype did not substantially differ from non-transgenic control plants in their main agrobiological characteristics when were grown under *in vivo* condition such as farming time, individual yield and

fruit quality. These results show a promising prospect for *ctb* antigen production by genetically engineered tomatoes.

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Contribution of Authors

Loc NH: Designed research methodology, processed and analysed experimental data and wrote the manuscript

Thinh LT: Conducted the research work, collected data and participated in manuscript write up