Genetic transformation of tomato (*Lycopersicon esculentum* Mill.) with *Agrobacterium rhizogenes* and production of 1ycopene in transformed root culture

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Different cell density of *Agrobacterium rhizogenes* ATCC 15834 and inoculation time were tested to investigate the ability for transformation and production of 1ycopene in *Lycopersicon esculentum* Mill. *Agrobacterim rhizogenes* used in this study were able to produce hairy roots. The transformation frequency was 33-59%. Bacterial cell density and inoculation time didn't affect on transformation frequency and root number per explants but affected growth and 1ycopene content of hairy root. The developed culture exhibited fast growth and high lateral branching on hormone-free liquid MS medium. Higher bacterial cell density OD and longer inoculation time decreased fresh weight of hairy roots. Cell density of *Agrobacterium* significantly affected 1ycopene content of hairy root while effect of inoculation length was non significant at 5% probability level. Cell density OD = 0.5 on all inoculation time produced 1ycopene content higher than that of cell density OD = 1.0. The highest fresh weight (1970 mg) and 1ycopene content (1.68 µg/g) was observed after 6 weeks of hairy roots culture produced by inoculation of *Agrobacterium* at cell density OD = 0.5 for 15 minutes. Lycopene content of root closely related to growth of hairy root.

Keywords: *Agrobacterium rhizogenes*, *Lycopersicon esculentum*, 1ycopene, hairy root, secondary metabolite, cell density, inoculation time.

INTRODUCTION

Lycopene is a source of carotenoid and has a strong antioxidant effect. They have a higher antioxidant activity than a beta-carotene and vitamin A, C, E, or the other mineral (Di Mascio *et al*., 2002; Heber and Lu, 2002). Result of *in vivo* research showed that 1ycopene could inhibit carcinogenic in animal and human cell (Stacewicz-Sapuntzakis and Bowen, 2005; Liu *et al*., 2006). Lycopene consumption had been proven could decrease the risk of some cancer and other degenerative diseases caused by oxidative stress (Giovannuci *et al*., 2005; Singh and Prasanth, 2012).

Lycopene is found in tomato, apricot, guava, watermelon, papaya, grape (Chalabi *et al*., 2004). Lycopene content in tomato is relatively higher than in other fruits or vegetables and 1ycopene input into the human body is at least 85 % come from tomato and tomato derived product (Bramley, 2000; Chalabi *et al*., 2004). However, tomato 1ycopene content varies considerably is affected by variety, maturity, and both agronomic and environmental conditions during growing (George *et al*., 2004; Kaur *et al*., 2006).

In the last decade, secondary metabolite production was changed to the use of hairy root culture technology. This technology has proven as an efficient alternative

production system to produce secondary metabolite on many plants (Giri and Narasu, 2000; Guillon *et al*., 2006). Hairy root induction can be done by using *Agrobacterium rhizogenes* vector. *Agrobacterium rhizogenes i*s gram negative soil bacteria and the causal agent for the development of the hairy root disease in plants. *Agrobacterium rhizogenes* has the ability to transfer a portion of its DNA (T-DNA) of root-inducing (Ri) plasmid to the genome of the plant (Nilson and Olsson, 1997). T-DNA will be integrated into plant chromosomes and express genes to synthesis opine substance and oncogene coding the growth hormone of auxin and cytokinin. Oncogene expression in Ri-plasmid is characterizing the adventives root formation abundantly at the infected sites recognized as hairy root (Nilson and Olsson, 1997).

The advantages of transformed hairy root culture in secondary metabolite production are relatively homogen, high genetic stability, and can be grown in a medium without any addition of growth regulator. Besides that, the growth of hairy root is faster than the root derived from induction with growth regulator, and is easily manipulated to increase the secondary metabolite production (Doran, 2006). Many medicinal plants have been transformed successfully by *A. rhizogenes* and the hairy roots induced show a relatively high productivity of secondary metabolites, including metabolite of hyoscyamine and scopolamine from *Hyoscyamus* (Akramian *et al*., 2008), anthraquinone from *Rubia cordifolia* cell (Bulgakov *et al*., 2003), and *Datura innoxia* (Biotel-Conti *et al*., 2000).

In this study, we investigated the effect of cell density of *A. rhizogenes* and inoculation time on induction of hairy root cultures of *L. esculentum* and evaluated 2ycopene production capacity and growth rate in hairy root culture.

MATERIALS AND METHODS

Plant Material

Seeds of tomato (*Lycorpersicon esculentum* Mill.) were surface sterilized with 2% sodium hypochloride and rinsed with sterile distilled water. The sterile seeds cultured on MS basal media (Murashige and Skoog, 1962) without any phytohormone for *in vitro* germination. Hypocotyls from 7-day old seedlings are used as explants for infecting with *Agrobacterium rhizogenes*.

Bacterial strain

Agrobacterium rhizogenes strain ATCC 15834 was used in the experiments. The strain was maintained and cultured on semi-solid Yeast Mannitol Broth (YMB) medium. The bacterial strain was grown in dark for 48 hours at 25 ± 2ºC temperature and were further maintained at 4ºC in refrigerator. Before using the bacterial strain for transformation experiment, bacterial suspension culture was prepared by transferring a single cell colony in liquid YMB medium in sterile culture tubes and incubated for 24-48 hours on rotary shaker at 100 rpm. The bacterial growth was determined by its optical density measured at 600 nm using spectrophotometer (Mehrotra *et al*., 2008).

Induction and production of hairy root via *A rhizogenes* transformation

Hypocotyls explants of 7 days old tomato seedling were wounded with metal needle and infected with *A. rhizogenes* strains ATCC 15834. Bacterial suspension of different optical densities (O.Ds) i.e, 0.5 and 1.0 at 600 nm were used for co-cultivation. The mixed suspensions of explants and *A. rhizogenes* were shaken at 70 rpm for 15, 30, 45 and 60 minutes. The inoculated hypocotyls were co-cultivated with *A. rhizogenes* for 3 days at 25ºC in the dark. After co-cultivation, the explants were washed with 500 mg L^{-1} cefotaxime antibiotic for 5 minutes, and were then cultured on the selection medium (MS medium) containing 500 mg L^{-1} cefotaxime antibiotic for 10 days to kill the bacteria. Explants were then transferred to hormone-free MS media and incubated at 25 ºC under light. Frequency of hairy root formation and root number per explants for each treatment was scored 4 weeks after co-cultivation. Four weeks after co-cultivation, hairy roots emerging from infected explants were excised and transferred to hormone-free liquid MS medium and shaken in orbital shaker at 100 rpm. The roots were sub cultured onto the same medium every 2 weeks for 8 weeks. Every 2 week, hairy roots from each treatment were weighed out and were analyzed for lycopene contents.

Analysis of Lycopene Content

Lycopene extraction method was done according to Perez, *et al*. (2008). Hairy root samples were grinded and homogenized. Samples of 0.6 g were added with 5 ml solution of 0.05% (w/v) butylated hydroxytoluen (BHT) in acetone, 5 ml ethanol and 10 ml hexane. Homogenate were put in ice and shaken with stirrer for 15 minutes, then were added with 3 ml deionized water on every vials and were shaken for 15 minutes in cold. Samples were left at room temperature for 5 minutes in order to separate the two phases. Qualitative analysis was done

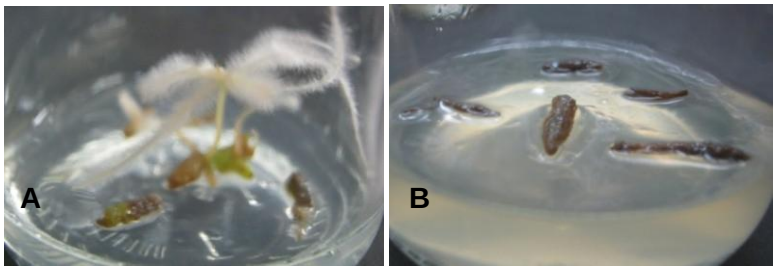

Figure 1. Development of hairy roots on hypocotyls explants of *Lycorpersicon esculentum* Mill. four weeks after inoculation with *A. rhizogenes* ATCC 15834 after incubation in MS medium containing kanamycin. A. Transformed hypocotyls, B Non-transformed hypocotyls.

by using Thin Layer Chromatography (TLC) to observe the existence of an active substance as lycopene in root extraction .Standard solutions used were lycopene (Sigma). TLC analysis was done by putting 20 µl concentrated root extract on silica gel plate 60 F_{254} using capillary pipette. Plate with dots of samples was immersed into eluen with the proportion of 75:25 (hexane:acetone) then was observed under UV at 366 nm λ. Lycopene content was analyzed with uv–vis spectrophotometer and was done by taking hexane phase solution (upper phase) and then being observed at 417 nm λ. Hexane solution was used as blank.

RESULTS AND DISCUSSION

Induction of Hairy Roots

Agrobacterium rhizogenes strain ATCC 15834 could induce the tomato root formation of hypocotyls explants. *Agrobacterium rhizogenes* ATCC 15834 induced hairy roots of tomato at a frequency between 36 and 59% of the explants and induced an average of 1-2 hairy roots of explants. Hairy roots were emerged at the infection sites or wounded regions of the hypocotyls explants at 7-10 days after co-cultivation with *A. rhizogenes*. Hairy roots displayed a typical phenotype characterized by grow faster, have reduced apical dominance, which produces highly branched roots and the roots were plagiotropic (Figure 1A). Conversely, none of the explants uninfected *A. rhizogenes* produced roots (Figure 1B). In some species non-inoculated explants produced roots, but these non-transformed roots showed poor growth and rare branching compared to the much more vigorous growth of the hairy roots (Koike *et al*. 2003; Christensen *et al*. 2008). In this way, hairy roots can be distinguished from non-transformed roots and selected based on their hairy root phenotype as the primary indicator of transformation without using antibiotic or herbicide resistance genes for selection (Christensen *et al*., 2008).

Statistical analysis revealed that cell density of *Agrobacterium* and inoculation time didn't significantly affect on induction of hairy root. There were no difference among treatments in transformation frequency and root number per explants in this experiment, but there was a tendency to decline in transformation frequency (Figure 2A) and roots number (Figure 2B) on higher cell density OD and longer inoculation time. Maximum transformation frequency (59%) was observed at OD =1.0 with 15 minutes inoculation time, while the highest root number was observed at OD = 0.5 with 30 minutes inoculation time.

These results were supported by Rashid *et al*. (2011) and Hu *et al*. (2003) who used *Agrobacterium* cell density OD = 0.5 and obtained maximum transformation efficiency. In case of higher OD, transformation efficiency was lower. Higher optical density (OD>0.6) decreased transformation frequency of *Lotus corniculatus* (Jian *et al*., 2009). It is because of the contamination could not be controlled and excessive bacterial growth was observed at these higher level of bacterial culture, as a result of which explants died. While Karthikeyan *et al*. (2007) obtained the frequency of root formation and number of root per explants of *Arachis hypogaea* L. was reduced with increasing infection period. Similar observations about the length of the co-cultivation period have been reported by others (Viemont and Lambert, 1994; Rahimi *et al.*, 2008; Jian *et al*., 2009). Longer co-cultivation periods did not improve production of hairy root of *Valeriana sisymbriifolium* (Rahimi *et al*., 2008). The similar trend was already reported in *Ericax darleyensis* (Viemont and Lambert, 1994). According Jian *et al*. (2009), longer co-cultivation time caused the overgrowth of *A. rhizogenes* leading to damage of the plant cell and consequently resulting in low transformation frequency.

Hairy roots are induced when a plant is infected by an *A. rhizogenes*, by a part of DNA (T-DNA) of root inducing (Ri) plasmid transferred into the plant cell and expressed

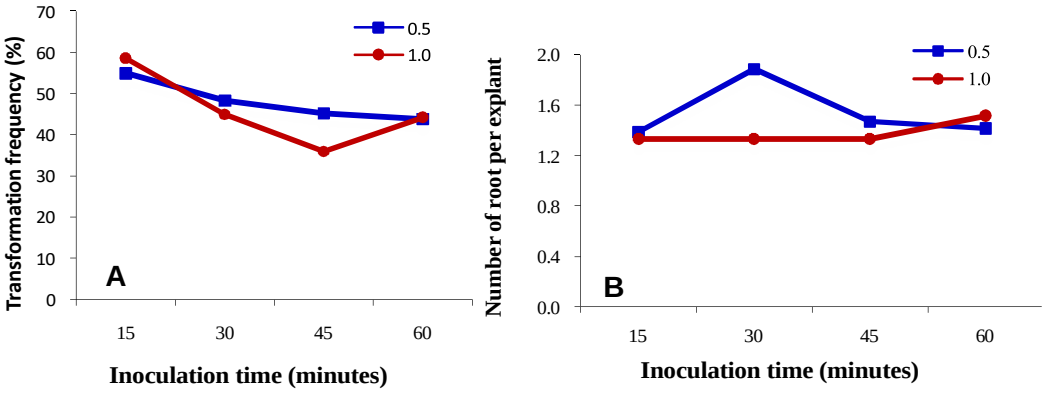

Figure 2. Effects of bacterial cell density OD and inoculation time on hairy root growth from tomato hypocotyls explants (*Lycopersicon esculentum* Mill.) transformed with *A.rhizogenes.* A. Transformation frequency, B. Number roots per explant

Figure 3. Development of hairy roots from hypocotyl of tomato after inoculation with *A. rhizogenes* in hormone-free liquid MS medium.

therein. The T-DNA of the *A .rhizogenes* consists of the TL- and TR-DNA. According to Nilson and Olsson (1997), TL-DNA brought *rol* genes, i.e. *rol*A, *rol*B, *rol*C, and *rol*D. *Rol*A was a gene responsible to root growth, while *rolB* was the main factor in hairy root formation (Rhodes *et al*., 1994; Tenea *et al*., 2008). Accumulation of *rol*B transcript in *Glycyrrhiza* plants increased the root formation and transformation efficiency (Tenea *et al*., 2008).

A number of previous studies showed that genetic transformation mediated by *A. rhizogenes* was affected by explants genotype, bacterial strains (Dogan *et al*., 2005), bacterial cell density, and co-cultivation conditions (Jian *et al*., 2009; Rashid *et al*, 2011). Most plant tissue and organs, including hypocotyls, cotyledons, stems, leaves, petioles, shoots and roots have shown capacity to be infected by *A. rhizogenes* resulting in production of hairy roots (Dogan *et al*., 2005; Karthikeyan *et al.*, 2007; Triplett *et al*., 2008; Kim *et al*., 2010; Pirian *et al*., 2012). However, within a plant species, the different organs differ in susceptibility to *A. rhizogenes* infection, since the response varies depending upon the *A. rhizogenes* strain and its interaction with the plants genes (Christensen and Muller 2009; Pirian *et al*., 2012). According to Akramian *et al*. (2008), different strains of *A. rhizogenes* vary in their transforming ability because of the significant differences in virulence. While Jian *et al*. (2009) stated that the growth status of *A. rhizogenes* may influence its virulence, and thereby the transformation frequency. The highest transformation frequency of *Lotus corniculatus* (Jian *et al*., 2009) and *Portulaca oleracea* (Pirian *et al*., 2012) were obtained when *A. rhizogenes* cultures at the late-log stage were used, corresponding to OD = 0.6. At this OD, transformation frequency increased significantly over all other tested cell concentrations.

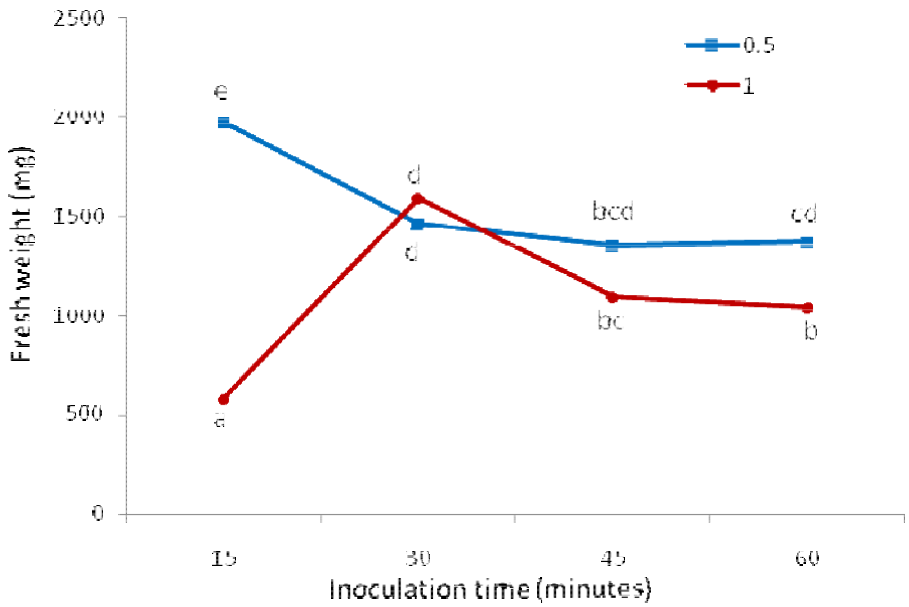

Figure 4. Effect of bacterial cell density and inoculation time on fresh weight of hairy roots after 6 weeks of culture in hormone-free liquid medium.

Growth of Hairy Roots in Liquid Culture

After 4 weeks on kanamycin-containing media, hairy root were transferred into hormone-free liquid MS medium to increase hairy root growth. Hairy root cultures were maintained in hormone-free liquid MS medium for 8 weeks and sub-cultured every 2 weeks. Transfer to liquid culture greatly stimulated new growth of the cultures. Even without addition of plant growth regulator in the medium, the roots were capable to grow fast (Figure 3). Fresh weights of the hairy roots in the liquid culture were significantly higher than their initial weights. It is indicated that roots which were cultured were transforman roots. *Agrobacterium rhizogenes* carries T-DNA that induces the formation of roots after its integration into the plant genome. According to Doran (2002), one of the advantages of using *A. rhizogenes* was an economic culture with unnecessary to add hormone, if compared to cell suspension and non-transformation root culture.

Statistical analysis showed that cell density of *Agrobacterium* and inoculation time significantly affected fresh weight of hairy root at 5% probability level. Higher bacterial cell density OD and longer inoculation time decreased fresh weight of hairy roots (Figure 4). Cell density OD = 0.5 resulted higher mean of root fresh weight compared to OD = 1.0. Hairy roots growth in response to bacterial infection at OD = 0.5 for 15 minutes attained maximum fresh weight (1970 mg). This result showed that inoculation with *Agrobacterium* at OD = 0.5 for 15 minutes effectively induced the hairy root formation on hypocotyls explants. The high cell density OD in this research resulted low root fresh weight may be due to a lot of bacterium number that made ineffectively infection of explants and inhibited hairy root growth. While the declining of root fresh weight with an extended inoculation time was probably due to accumulation of substance produced by bacterium that inhibited gene transfer from *A. rhizogenes* into plant cell. Karthikeyan *et al*. (2007) stated that 15 minutes infection time resulted highest hairy root production on peanut and the longer inoculation time the lower fresh root weight produced.

Determination of Lycopene Content of Hairy Roots

Thin layer chromatography using hexane:aceton eluen (75:25) with uv irradiation of 366 nm showed that hairy root sample spot with Rf values comparable to that of standard lycopene. These compounds showed blue fluorescence at λ 366 nm (Figure 5).

Statistical analysis revealed that cell density of *Agrobacterium* significantly affected lycopene content of hairy root while effect of inoculation time was non significant at 5% probability level. Cell density OD = 0.5 on all inoculation time produced lycopene content higher than that of cell density OD = 1.0. Maximum lycopene (1.68 µg/g) was observed after 6 weeks of hairy roots culture produced by inoculation of *Agrobacterium* at cell density OD = 0.5 for 15 minutes (Figure 6). Although inoculation time have no effect on lycopene content there

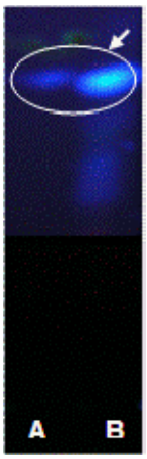

Figure 5. Thin Layer Chromatography (TLC) of extracts of transformed hairy root of tomato. A. standart lycopene; b transformed hairy root.

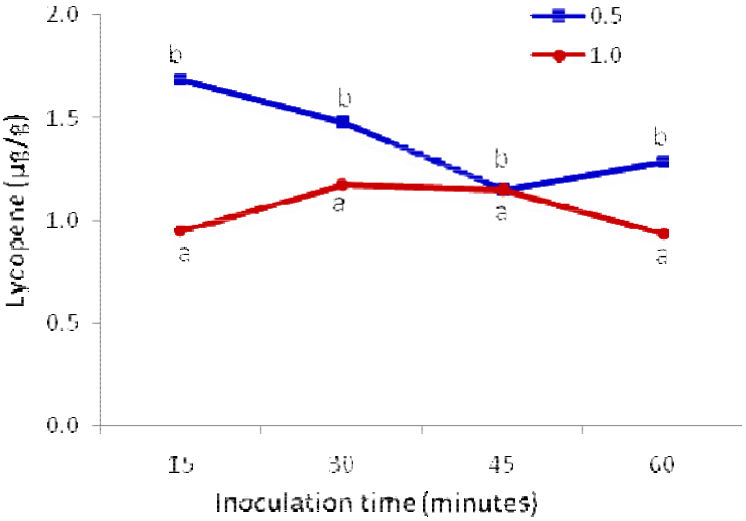

Figure 6. Effect of bacterial cell density and inoculation time on lycopene content of hairy roots.

was a gradual decline in lycopene content from 15 minutes inoculation time to 60 minutes on hairy roots was infected *Agrobaterium* at OD = 0.5. The reverse pattern was observed in case of cell density OD = 1.0, there was a gradual increase in lycopene content in inoculation time from 15 minutes to 45 minutes.

The high cell density OD in this research caused poor growth of hairy root growth. It has been suggested that inhibition of hairy roots growth caused inhibition on accumulation of lycopene content. Other possibilities due to high cell density infected more explants so most of secondary metabolite role as an antipathogen agent, and less lycopene was produced. Overall differences in the production of lycopene of hairy roots produced in response to infection *A. rhizogenes.* According to Bulgakov *et al*. (2008), *A. rhizogenes rol* genes having the role as modulators of cells growth and differentiation, but has found the new role of the genes when interacting with plants that is potentially as activators of secondary metabolism in the transformed cells. While according to

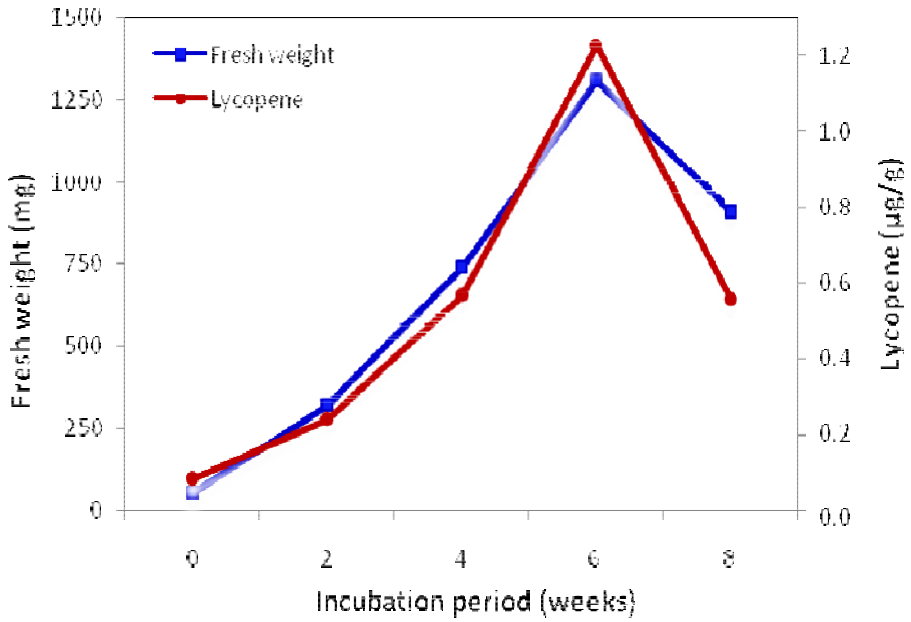

Figure 7. Relationship between growth and lycopene production from transformed hairy roots of *Lycopersicon esculentum* Mill.

Vu *et al*. (2006), infection of *A. rhizogenes* have a positive effect on growth of hairy roots and production of secondary metabolite was due to *A. rhizogenes* have role as biotic elicitor that increase alkaloid content and biomass of *Datura innoxia*.

Based on time course of growth and lycopene production showed that lycopene content appeared to be closely related to growth of hairy roots (Figure 7). There was an increase in growth and lycopene content with an increase in number of weeks of incubation. There was an increase in biomass from 55 mg at day one to 1307 mg at 6 weeks. Lycopene production increased also during the six weeks and reached a value of 1.23 µg/g. At 8 weeks, there was a sudden decrease in the lycopene content and fresh weight of hairy root. According to Tenea *et al.* (2008), secondary metabolite content of hairy roots correlated with fresh weight of roots. Giri *et al*. (2008) and Ahlawat *et al*. (2012) reported that increased roots fresh weight of *Artemisia* followed by increasing artemisinin content.

The present investigation emphasizes that hairy root can be induced from hypocotyls explants of *L. esculentum*. Proliferation of the hairy roots can be conducted in hormone-free liquid medium. Different cell density and inoculation time have no effect on induction of hairy roots but affected on growth and production of lycopene in hairy root culture. The highest fresh weight and lycopene content was observed after 6 weeks of hairy roots culture produced by inoculation of *Agrobacterium* at cell density OD = 0.5 for 15 minutes. Lycopene content of root correlated with growth of hairy root.

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