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Root Growth and Gossypol Content in Gossypium hirsutum L. Root and Hairy Root Cultures.

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Abstract

Experiment was conducted to optimize gossypol production as well as root and hairy root growth based on the concentration of LS medium. Hairy root was induced by Agrobacterium rhizogenes strain ATCC-15834. There were four treatment applied 1) Full-strength LS medium for normal roots culture (LNT), 2) Full-strength LS for hairy roots culture (LT), 3) Half-strength LS for normal roots culture (1/2NT), 4) Half-strength LS for hairy roots culture (1/2T). All treatment produced the highest root growth on days 20th, but the highest dry weight of roots was obtained at 1/2T treatment. The highest gossypol content in roots was obtained on days 16th for all treatments and all were growth associated, while the amount of gossypol that was secreted into culture medium were obtained on days 20th for all treatments. Highest gossypol content was also achieved in 1/2T, both in hairy roots and medium. According to these results, it could be concluded that 1/2T represented the best treatment combination for growth as well as gossypol production in root and medium.

Keywords— Gossypium hirsutum L., Root growth, Gossypol, Hairy root, LS concentration.

Introduction

Gossypol is a di-sesquiterpene yellowish pigments, present in various parts of cotton plants (Marchlewski,1899). It exhibits multiple biological properties, including male anti fertility activity (Endress, 1994), anti tumor activity (Duke, 1987) and insecticidal activity (Rogers, 1995). Gossypol in the cottonseed can also be used as dietary supplement in broilers to increase their weight (Bailey *et al.*, 2000). In the past few decades, effort have been made to produce valuable plant metabolites using different biotechnological tools, among them is the application of *in vitro* culture techniques, i.e. by growing plant tissues, organs or undifferentiated cells, which will then be used to induce secondary metabolite synthesis and accumulation, by not using the plant *in vivo* (Zarate, 1999).

The synthetic capacity of dedifferentiated tissues often differs substantially from that of fully differentiated tissue, both quantitatively and qualitatively (Suzuki *et al.*, 1987). A number of attempts have been made to produce gossypol by callus culture, however its production ability was not satisfactory. Yulia (1999) even found low gossypol content in callus culture of *Gossypium hirsutum* L. which was elicitated by *Rhizoctonia solani*.

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Many important plant secondary metabolites are synthesized in root tissues which can then be stored *in situ* or transported to other plant organs (Waller and Nowacki, 1978). Gossypol is abundance in the roots of *G. hirsutum* L. (Nomeir and Abou-donia, 1982). Recently, the roots of several species transformed by *Agrobacterium rhizogenes* were capable of quite rapid growth in *in vitro* defined nutrient medium without the requirement for exogenous phytohormones (Tepfer and Tempe, 1981; Wilmitzer *et al.*, 1982). Therefore, increasing of gossypol content in plant organ culture could be conducted by using hairy roots culture technique transformed by *A. rhizogenes* (Drewes and Staden, 1995). Several studies on the biosynthesis of the secondary metabolite using hairy root cultures showed that tropane alkaloid was produced in the *Hyoscyamus muticus* culture (Flores and Filner, 1985), tropane alcaloid in the *Atropa baetica* culture (Zarate, 1999) and puerarin in the *Pueraria phaseoloides* culture (Shi and Kintzios, 2003).

Changes in hairy root metabolite formation and morphology were influenced by physical and chemical stimuli, including nutrient limitation, illumination, and hormone concentration (Tone *et al.*, 1994a, 1994b). Some researches on the influence of medium to the production of secondary metabolite in the hairy root culture have been conducted. In the culture of red beet (*Beta vulgaris*) hairy roots, phosphate is the key nutrient for increasing the accumulation of betanin pigment in the roots. Normal root growth, which was parallel with enhanced pigment formation, was achieved even in medium without phosphate (Taya *et al.*, 1994b). Flota *et al.* (1994) found high biomass of root *Catharanthus roseus* with specific nitrate/ammonium ratio. Higher tropane alkaloids was achieved in hairy roots of *A. baetica* transformed by *A. rhizogenes* strain ATCC-15834 cultivated in half Murashige-Skoog medium (Zarate, 1999). Pramudiyanti and Rizkita (2004) reported that there were parallel relationship between root growth and gossypol content in the root culture of *G. hirsutum* L. cv. tamcot. Therefore, this research will study the effect of a half and full-strength nutrition concentration of Linsmaier-Skoog (LS) medium in order to get optimal root growth and gossypol content in *Gossypium hirsutum* untransformed and hairy root culture.

Research Methods

Hairy root induction

Agrobacterium rhizogenes strain ATCC-15834 (48 h, old) was used to initiate hairy root on *G. hirsutum* L. cv. kanesia 7 culture, cultured on LS full-strength solid medium. Cultures were decontaminated with cefotaxime followed by culturing in the dark for 30 days in LS agar medium supplemented with cefotaxime. The hairy roots emerged and confirmed 7-10 days after infection (Rizkita and Wardani, 2003). Hairy roots were excised from the explants and transferred to flasks containing either half or full-strength concentration of LS liquid medium. The culture flasks were then placed on an orbital shaker at 120 rpm in dark condition and temperature of 26±2ºC (Kusumadewi, 2004). The established hairy root culture was sub-cultured every 10 days. Three replicate flasks from each nutrient medium were harvested every 4 days over a culture period of 36 days to determine the root growth (dry weight) and gossypol content from both hairy roots and liquid medium

Root (nontransformed) culture

Non-transformed roots were obtained from 3 days old sterile seedlings of *G. hirsutum* L. cv. kanesia 7. Roots were cut ±2cm from the tip and cultured in two different liquid media (LS medium with half and full-strength concentration). The culture conditions were the same as the hairy roots culture conditions. (Nurchayati and Rizkita , 2006).

Roots growth parameter

Time course of growth was observed by sampling roots every 4 days during the culture cycle of 36 days, and dry weight was evaluated as parameter of root growth

Extraction and determination of gossypol

The quantification of gossypol in hairy roots and non-transformed roots was conducted by tissue extraction using modified Nomeir and Abou-donia (1982) method. The extract was separated by reversed phase high-performance liquid chromatography (HPLC from Shimadzu Ltd., Japan) using a Shimpack VP-ODS C-18 (4.6 mmx150 mm) coloumn in a solvent system of CH_3OH:H_2O (9:1) isocratic, by adding 0.1% phosphoric acid, at λ 230 nm. Standard gossypol was purchased as gossypol-acetic acid from sigma, its purity was determined by HPLC to be 99.5%. Data processor was a chromatopac CR-7A plus (Shimadzu Ltd., Japan). The data processor was used to measure the retention time, peak areas, and the percentage of each peak in the chromatogram.

Results and Discussion

Root Growth

Hairy roots was successfully initiated in solid and liquid LS medium (Figure 1a and b), while nontransformed (normal) root was in LS liquid medium (Figure 2). The result showed that nutrition concentrations influenced the root growth pattern (Figure 3a and b). Roots entered the growth phase immediately and achieved highest dry weight on days 20th (late logarithmic phase) for all treatment combinations. Highest dry weight of roots was obtained on 1/2T treatment combination (0.1740±0.0036 gDW). Taya *et al.* (1994) found a normal root growth and formation of betanin in medium without phosphate.

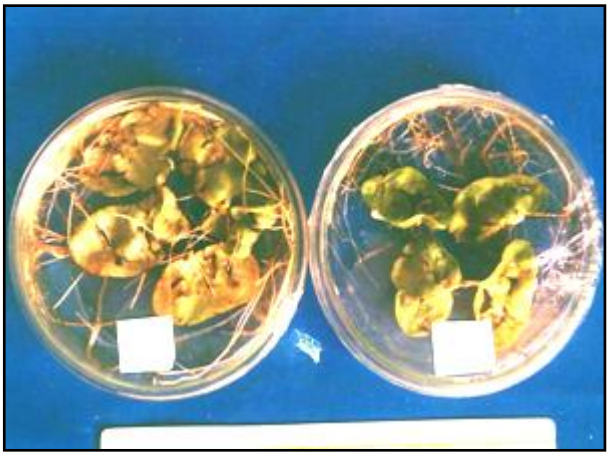

Figure 1(a). Hairy roots (30 days), cultured in solid LS medium.

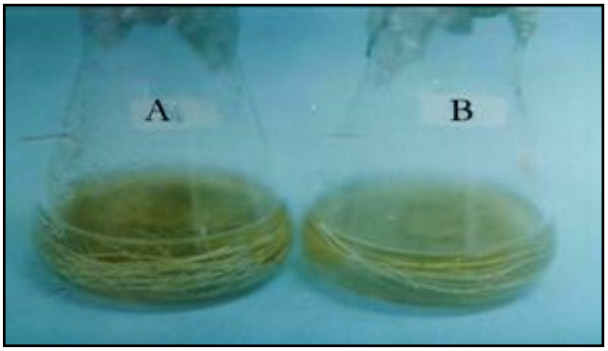

Figure:1(b) Hairy roots in liquid LS medium.

A half-strength nutrition concentration (A), Full-strength nutrition concentration (B).

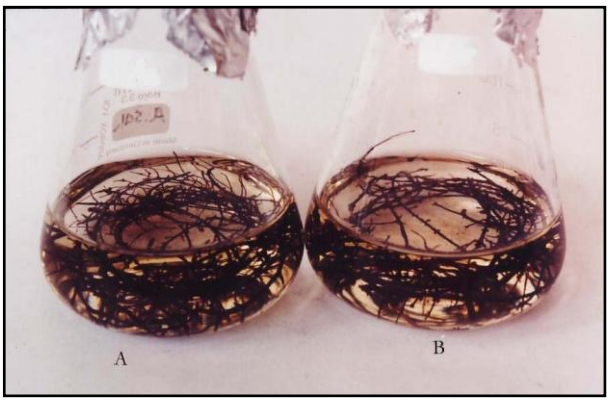

Figure:2. Normal roots culture.

A half-strength nutrition concentration (A), Full-strength nutrition concentration (B).

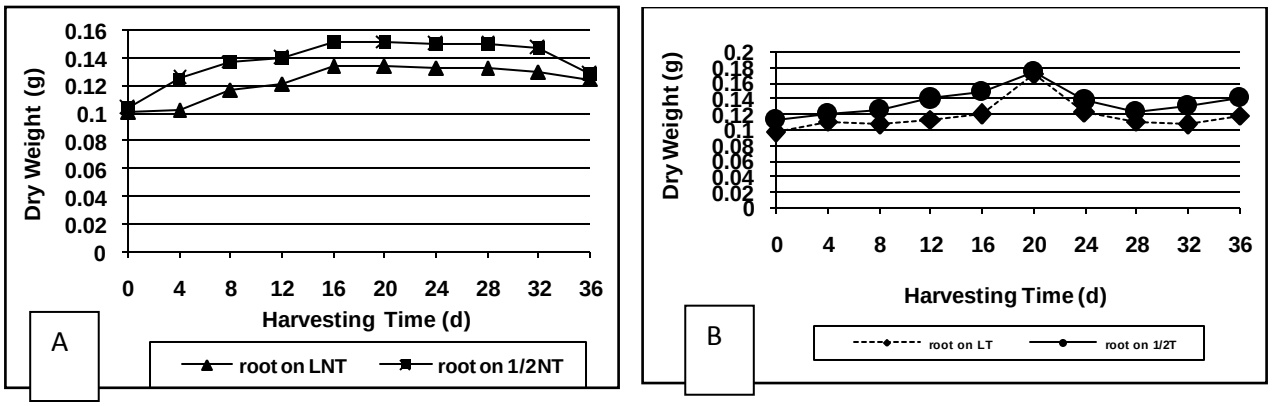

Figure:3. Root growth pattern in normal root cultures (a) and hairy root cultures (b).

Results of this experiment showed that dry weight of roots on treatment combinations of a full-strength nutrition concentration (LT, LNT) was lower than that on a half-strength nutrition concentration (1/2NT,1/2T), data was presented in Table 1. Muranaka *et al.* (1992) reported that high nitrate (NO_3^-) concentration in KNO_3 caused several toxic effects in the root cultures physiological conditions. Franklin and Dixon (1994) also reported that concentration of KNO_3 more than 15 mM in MS medium could reduce rapid growth in the culture. It was indicated that low ammonium (NH_4^+) and nitrate (NO_3^-) ratio was favorable to roots growth.

Nutrition concentration caused a significant effect on root growth, while the type of root factor and interaction between the two factors did not reveal significant effects. These results indicated that root growth patterns in normal roots culture and hairy roots culture were similar.

Table 1:

The effect of nutrition concentration and root type on root dry weight for all treatment combinations.

Treatment	Dry Weight (g)
(LNT)	0.1224±0.0137 a
(1/2NT)	0.1382±0.0187 b
(LT)	0.1171±0.0203 a
(1/2T)	0.1351±0.0175 b

Bulgakov *et al.* (2002) investigated carbohydrase enzyme activity that caused *rolC* gene insertion in *Panax ginseng* culture, and found obvious result from his research, that *rolC* gene could affect carbohydrase enzymes activity such as 1,3-β-D-glucanase. However, authors suggested that 1,4-β-D-glucanase enzyme expression was suppressed, therefore root growth (hairy roots) was delayed.

Gossypol content in roots

The highest gossypol content in roots was obtained on days 16^{th} for all treatment combinations (Figure 4). Based on statistical analysis, it was found that nutrition concentration caused a significant effect on gossypol content in roots, while type of root factor did not show a significant effect. Interaction between the two factors revealed a significant effects. These results indicated that the two factors could be applied together, so that treatment with type of root factor could be elevated by nutrition concentration factor. 1/2T treatment combination represented the best treatment combination and significantly different to other treatment combinations (12.6479±0.2992 mg/gDW) (Table 2).

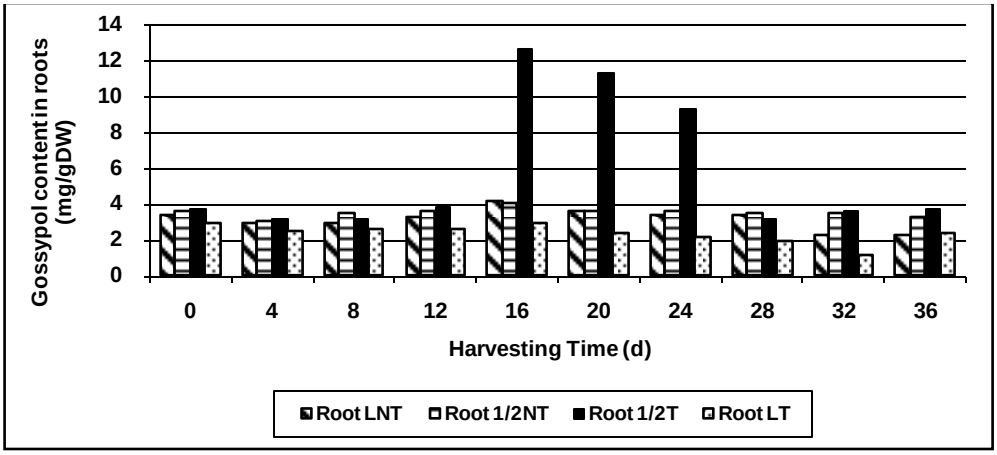

Figure :4. Gossypol content in roots for all treatment combinations. L: Nutrition concentration (Full-strength), ½: (Half-strength), T: Transforman roots, NT: Non transforman roots.

Table 2.

The effect of nutrition concentration and root types on gossypol content in roots for all treatment combinations. Data were transformed to logarithmic (^{10}log).

Treatment	Gossypol content in roots (mg/gDW)
(LNT)	0.4854±0.0862 b
(1/2NT)	0.5421±0.0467 b
(LT)	0.3552±0.1161 a
(1/2T)	0.6963±0.2334 c

This result was similar to Zarate (1999) which found high accumulation of atropine and scopolamine in hairy roots culture of *Atropa baetica* that were cultured in a half nutrition concentration of MS medium. Baiza *et al.* (1998) investigated hyoscyamine content in normal roots culture of *Datura stramonium* which found high hyoscyamine content on days 16th (logarithmic phase) using half nutrition concentration of MS medium. Khan and Harborne (1990) reported that low concentration of potassium (K) could increase alkaloid tropane synthesis in *Atropa acuminata* hairy roots culture. Higher alkaloid accumulation also occurred when nitrogen supply was lowered. According to Hammond-Kosack and Jones (1996), synthesis of phytoalexins secondary metabolite such as terpenoid (gossypol) and flavonoid was a consequence from plant-pathogen interaction. Bulgakov *et al.* (2003) investigated key processes of defense reactions in *Rubia cordifolia* callus culture which was transformed by *Agrobacterium rhizogenes* contained rolB and rolC genes. That result indicated a higher increment of anthraquinone synthesis in transformed culture compare to that of non transformed culture.

Significant positive correlation (r=0.725) between means of gossypol content in roots and dry weight at 1/2T treatment combination indicated parallel relationship i.e gossypol content in roots increased when dry weight of roots was also increase, and conversely. According to Endress (1994), Pramudiyanti and Rizkita (2004), Rizkita and Wardhani (2003), and Nurchayati and Rizkita (2006), gossypol accumulation in roots culture was parallel to dry weight.

Gossypol secretion into culture medium

The highest gossypol production into culture medium were obtained on days 20th for all treatment combinations (Figure 5). Statistical analysis showed a significant effect of nutrition concentration factor, type of root and interaction between the two factors to the gossypol secretion into culture medium. 1/2T treatment combination represented the best treatment combination, gossypol was secreted more into culture medium (11.4740±0.3304 mg/L) (Table 3).

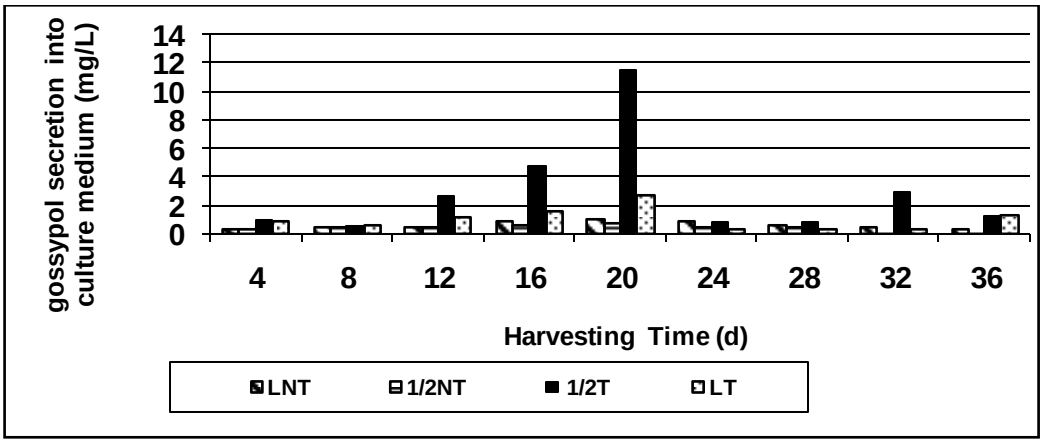

Figure 5. Gossypol secretion into culture medium for all treatment combinations. L: Nutrition concentration (Full-strength), ½: (Half-strength), T: Transforman roots, NT: Non transforman roots.

Nussbaumer *et al.* (1998) reported that hairy roots culture of *Datura candida* X *Datura aurea* cultured in a half-strength nutrition concentration of B5 (normal ratio of nitrate/ammonium: 25/2) medium secreted higher tropane alkaloid into culture medium compare to the medium with nitrate/ammonium ratio such as 25/5; 37/2; 50/2; 75/2; 40/2. So nitrate/ammonium optimal ratio influenced secondary metabolite production. The highest secretion of gossypol into culture medium was achieved on 1/2T treatment combination at pH of medium 4.57 (11.4740±0.3304 mg/L) (Figure 6). At the lowest pH of medium (3.45) secretion of gossypol was c.a 2.8764±0.0591 mg/L. However, Alvarez and Giuletti (2000) found that the highest alkaloid secretion into culture medium of the root culuture of *Brugmansia candida* was at 4.5 pH of medium, not at the lowest pH of a medium (3.5). Therefore they concluded that there was other mechanism which influenced the secretion of metabolite beside of pH in medium and cell necrosis.

Table :3.

The effect of nutrition concentration and root types on gossypol secretion into culture medium. Data were transformed to logarithmic (^{10}log).

Treatment	Gossypol in medium (mg/L)
(LNT)	0.7282±0.1988 a
(1/2NT)	0.6026±0.1720 a
(LT)	0.9034±0.3257 b
(1/2T)	1.2511±0.4123 c

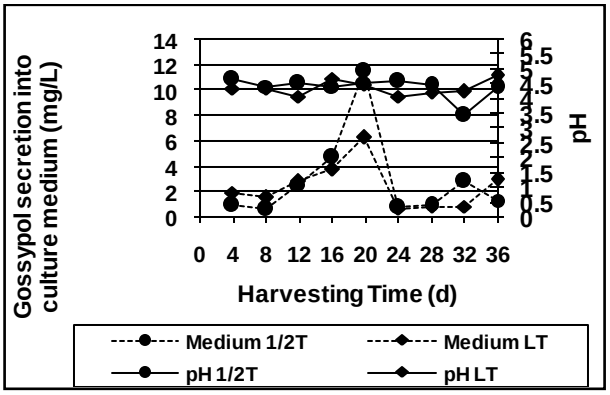

Figure 6. (a) Gossypol secretion into culture medium and pH of medium pattern in the hairy roots culture (1/2T, LT). L: Nutrition concentration (Full-strength), ½: (Half-strength), T: Transforman roots.

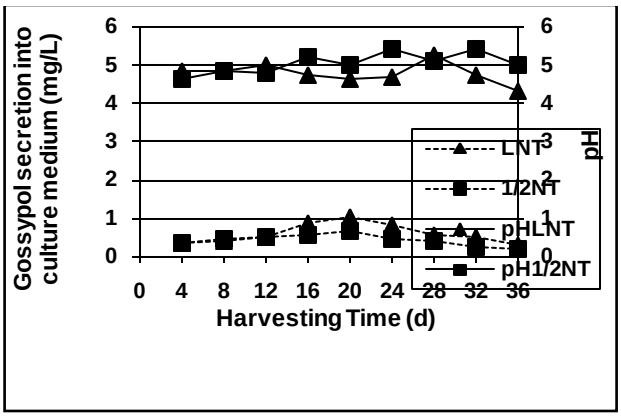

Figure 6. (b). Gossypol secretion into culture medium and pH of medium pattern in the normal roots culture (1/2NT, LNT). L: Nutrition concentration (Full-strength), ½: (Half-strength), NT: Non transforman roots.

Conclusion

1/2T treatment combination represented the best treatment combination. The highest Gossypol content in all roots type was achieved on days 16th. There was a parallel relationship between gossypol content in roots to growth of roots (dry weight). The highest dry weight of roots occurred on days 20th. Roots growth was affected by nutrition concentration. Gossypol was secreted into culture medium in the largest amount on days 20th. Hairy roots culture released more gossypol into culture medium compare to that in normal untransformed roots culture.

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