Journal of Sustainability Science and Management 2007 Volume 2 (2): 55-63

PRODUCTION OF TROPANE ALKALOIDS BY TRANSFORMED HAIRY ROOT CULTURES OF *Datura metel*

*[1]AZIZ AHMAD AND [2]MARZIAH MAHMOOD
[1]*Department of Biological Sciences, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu.*
[2]*Department of Biochemistry, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.*

**Correspondence authors:*
Email : aaziz@umt.edu.my Phone: 6096683179 Fax : 6096694660

Abstract: There remains a need to increase alkaloid production rates to favor their commercial exploitation as the growth and production of secondary metabolites is strongly influenced by the environmental factors. *Datura metel* transformed hairy roots obtained through infection with *Agrobacterium rhizogenes* LBA 9402, were employed for production of tropane alkaloids *in vitro*. The production of hyoscyamine and scopolamine by the hairy root cultures were investigated on three types of media at different ionic strength and sucrose concentrations. Roots cultured in B5 medium were observed to exhibit better growth and tropane alkaloid production compared to MS and White's medium. The full strength of B5 medium supplemented 4 % (w/v) of sucrose was yields the highest roots mass (550 mg dry wt. per flask), as well as the hyoscyamine and scopolamine which was 1.52 and 0.32 mg/g dry wt. of tissue, respectively. This indicated that, mineral nutrient and sucrose play an important role in tropane alkaloids biosynthesis by hairy root cultures.

KEYWORDS: hyoscyamine, scopolamine, *Agrobacterium rhizogenes,* transformation

Introduction

Higher plants produce diverse ranges of pharmaceutically important secondary metabolites. Different species of Solanaceae are rich sources of tropane alkaloids, mainly hyoscyamine and scopolamine which is widely used for their mydriatic, antispasmodic, anticolinergic, analgesic and sedative properties (Strauss, 1989). *Datura metel* a solanaceous plant, which is endemic to Malaysia, produces considerable levels of tropane alkaloids with anticholinergic activity (Aziz et al, 2002). Studies related to growth and tropane alkaloid production by transformed hairy root cultures of other *Datura* species have been publish earlier (Baiza et al., 1999; Nussbaumer et al., 1998). Other plants that have been initiated the transformed hairy root cultures for production of tropane alkaloid are including *Atropa, Brugmansia, Duboisia, Hyosycamus,* and *Scopolia* (Aziz, 2001). Generally, undifferentiated cell cultures do not produce these compounds efficiently due to biosynthesis of hysocyamine and scopolamine is correlated with root differentiation (Endo and Yamada, 1985) where they are synthesized in the pericycle of the roots (Yamada and Tabata, 1997). Furthermore, hairy root cultures have several advantages over normal ones, such as higher growth rates in phytohormones-free medium (Aziz et al. 2002), genetic and biochemical stability (Baiza et al., 1999) and amenable to genetic manipulation (Berkov et al., 2006).

Large-scale cultures of transformed hairy roots have been reported, however, in most cases, the yield of the tropane alkaloid hyoscyamine and scopolamine is too low for commercialization. There remains a need to increase alkaloid production rates to favor their commercial exploitation. Among the factors contributed on the yield including media and ionic strength (Nussbaumer et al., 1998), carbon sources (Rothe and Drager, 2002), nitrate and ammonium balance (Nussbaumer et al., 1998), plant growth regulators (Vanhala et al., 1998), feeding precursors (Aziz et al., 2002) and elicitors (Kang et al., 2004). Furthermore, the growth and production of secondary metabolites is

strongly influenced by the environmental factors than genetic ones (Berkov et al., 2006). In this paper we report on the effect of media and level of sucrose on the production of scopolamine and hysocyamine by *D. metel* transformed hairy root cultures.

Material and Methods

Transformed Root Cultures

Established transformed hairy root cultures of *Datura metel* as previously described by Aziz et al., (2002) were used in this study. The hairy roots were maintained in phytohormone-free B5 (Gamborg et al., 1968) liquid medium supplemented with 3% (w/v) of sucrose. The cultures were incubated in 28 ± 0.5 °C under the dark condition, vigorously shaking at 100 rpm on an orbital gyratory shaker. Subcultures were done every two weeks.

Treatments Medium

The production of tropane alkaloids by *D. metel* transformed hairy roots were investigated in three culture media which were B5, MS (Murashige and Skoog, 1962) and White's (White, 1934). All media used were phytohormone-free, supplemented with 30 g/L (w/v) sucrose and was adjusted to pH 5.7 prior sterilization by autoclaving at 121°C for 15 min. Consequently, the best medium from this experiment were chosen for further refine. The mediums were diluted into half and quarter strength, with full strength used as control. These media were added with various concentration of sucrose which was 1 to 8 %. A 0.2 g fresh weight of transformed roots was aseptically sub-cultured into 25 ml of respecting medium and cultivated as above for 30 days or otherwise indicated. Five replicates were used for each treatment. The harvested transformed roots were washed with de-ionized water to remove all the retained media and blotted dry between two layers of absorbent paper prior to the measurement of the fresh and dry weights.

Scopolamine and Hyoscyamine Analysis:

Dried tissues were powdered and extracted twice in Soxhlet for two hours using 250 ml of alkaline chloroform. The solvents were then dried at 40 °C under vacuum. The crude extract was dissolved in 25 ml chloroform and extracted twice or three times with 25 ml 2 % (v/v) of sulfuric acid. The organic phase was discarded, while the aqueous phase was made alkaline (pH 9-10) with ammonium hydroxide solution and extracted twice with 100 ml of chloroform. The chloroform fraction was then dried at 40 °C under vacuum and the tropane alkaloids were recovered. The composition of hyoscyamine and scopolamine was determined using HPLC as previously described (Fliniaux et al., 1993). The alkaloids was treated with 1ml of mobile phase at 40 °C for 15 minutes and passed through a Sep-Pak C18 solid phase extraction column (Waters, USA). A 20 µl of eluent was then subjected into NOVA-Pak C18 column and eluted with 12.5 % (v/v) acetonitrile and 0.3 % (v/v) phosphoric acid, pH 2.2 adjusted with trimethylamine (HPLC grade). The flow rate was 1.0 ml/min compound was monitored by Waters 501 HPLC detector at 204 nm.

Results and Discussion

Effect of Basal Media:

The effects of three different basal media on growth and tropane alkaloid content were examined after 25 days of culture. Among, the three media used, B5 medium was found to be the best for growth of *D. metel* transformed hairy roots (Figure 1a) and the tropane alkaloids production (1b). The growth of transformed hairy roots in B5 medium was 2.4-fold and 1.4-fold higher than in White's and MS media, respectively. Growth transformed hairy root cultures of hybrid of *Datura candida x D. aurea* was also strongly affected by different culture medium, whereby the maximum growth was obtained in a half-strength of B5 medium (Nussbaumer et al., 1998). Although, B5 was the best medium for *Datura* root cultures, the ionic strength was the major factor contributed to the proliferation of root cells. This results showed that the nutrient requirement for growth of hairy root is species dependent. Figure 2, showed growth of *D. metel* transformed hairy root cultures in B5 medium at every five days interval. There was a sharp increase in root growth between 5 to 15 days and was decreased after 15 to 25 days, gradual and observed to decrease at 30 days of cultures. The decrease in roots mass could be attributed to depletion of nutrients in the media. The optimum growth was observed at 15 days which was 7.6- fold higher then initial inoculums.

Hyoscyamine was the most abundant compared to scopolamine in roots of all media tested (Figure 1). Hyoscyamine production in B5 medium was 1.2 and 6.6-fold higher than that obtained in MS and White media, respectively. Scopolamine production was also higher in B5 medium which were 1.4-fold higher than MS, and 7-fold higher then in White's medium (Figure 1b). A small amount of hyoscyamine and scopolamine was found diffused into the media (Figure 1c). The amount of tropane alkaloids released into the culture medium was dependent on its production of root cultures. Excretion of alkaloids into B5 medium might be the consequence of higher biosynthesis process in the root tissues due to limited storage space in the tissue. The biosynthesis occurred in the pericycle of the roots (Yamada and Tabata, 1997) and supposed to have been transported to aerial part of plants. According to Yeoman and Yeoman (1996) the recovery of alkaloids from the media might be derived from the damaged and dead cells. However, the amount of alkaloids released into culture media remained very low compared to it total yields; the highest was 8.5 µg for hyoscyamine and 3.5 µg for scopolamine per culture of B5 media. This phenomenon was also recovered in *D. stramonium* transformed hairy root cultures as reported by Maldonado-Mendoza et al., (1993).

Figure 2b shows the scopolamine and hyoscyamine production in B5 medium at every five-day interval. Result shows that the yield of alkaloid increased with the biomass. Hyoscyamine and scopolamine level were highest at stationary phase, which was at day 25 with 1.95 and 0.36-mg/g dry wt. of culture, respectively. Similar observation was also obtained on *D. stramonium* transformed hairy root cultures by Maldonado-Mendoza et al., (1993). According to Yeoman and Yeoman (1996) low alkaloid yield at the early phase of growth is possibly due to the use of energy for enzyme activities for cell division. Moreover, the declined of scopolamine production after 25 days of culture might be due to limitation of oxygen. Oxygen atom was required in the process of epoxidation hyoscyamine to scopolamine by hyoscyamine 6β-hydrolyase enzyme (Hashimoto and Yamada, 1997).

Effect of B5 Strength

The effects of different sucrose levels (1 - 8 %) in different ionic strength of B5 medium on roots growth and alkaloid production are shown in Figure 3. The highest roots growth was obtained in full strength of B5 medium added with 4 % (w/v) of sucrose which was 550-mg dry wt/culture. This

was 2-times higher than that of roots cultured in half and quarter strength of B5 medium containing the same concentration of sucrose. Growth of roots in full and half strength of B5 media dropped sharply as the sucrose concentration in the medium was higher than 5 %. On the other hand, the growth of roots in quarter strength of B5 medium increased gradually as sucrose levels increased and the highest biomass was obtained at 5 % (w/v) of sucrose. The biomass of roots in half and quarter strength of B5 medium appeared lower than that of roots cultured in a full strength of B5 medium at all levels of sucrose tested (Figure 3). This study revealed that moderate concentration of sucrose which were between 3 to 5 % is suitable for growth of *D. metel* root cultures and it is dependent on the ionic strength. Our result was a contrast to *Rubia tictorum* transformed root cultures which showed high level of sucrose up to 12 %, (w/v) required to increase the biomass (Sato et al., 1991). Study on *Datura candida x D.* aurea transformed hairy root cultures root by Nussbaumer et al., (1998) shows that the roots growth was faster (2- fold) when cultured in B5 medium supplemented with 5 % (w/v) of sucrose. In *Hyoscyamus albus* transformed hairy root cultures, the biomass was not strongly affected by the sucrose concentration (Christen et al., 1992). Suggesting, the sucrose requirement for growth of hairy roots is species dependent.

Sucrose concentration was also observed greatly to influence the scopolamine and hyoscyamine produced by *D. metel* transformed root cultures. The highest scopolamine and hyoscyamine production was obtained in medium added with 4 % (w/v) of sucrose at all strength of B5 media used. Full strength medium was produced higher hyoscyamine and scopolamine content than half strength and quarter strength of B5 media which was 2-fold and 3-fold, respectively (Figure 3b and 3c). This result was contrast with study by Sauerwein and Shimomura (1991) on *H. albus* transformed hairy roots which the highest tropane alkaloid was obtained in medium with lower sugar content (2 % w/v). In *Centranthus rubber* transformed hairy root cultures showed that half strength of B5 medium supplemented with 3 % (w/v) of sucrose produced the highest yield of valepotriate (Granicher et al., 1995). Meanwhile, in hybrid of *D. candida x D. aurea* transformed hairy root cultures the scopolamine and hyoscyamine content did not differ significantly in media supplemented with 4 to 7 % (w/v) of sucrose (Nussbaumer et al., 1998). This indicated that, in the influence of sucrose on the biosynthetic capacity or hyoscyamine/scopolamine ratio is species dependent and might be under genetic control. Furthermore, according to Kanagae et al., (1994) the expression of the hyoscyamine-6-hydroxylase gene in the pericycle is species dependent.

Conclusion

It could be concluded that the growth and the production of tropane alkaloids is species dependent. Ionic strength and sucrose concentration are among the factors that influence the growth as well as the yield of alkaloids. Full strength of B5 medium was the best medium for biomass and tropane alkaloids by *D. metel* transformed hairy root cultures. The hairy root required minimum 15 days to achieve the optimum growth rate and sustained for 25 days prior to its death. Alkaloids production was also increased accordingly to the growth of roots. Further studies are needed to determine the most functional ions in the growth of hairy roots and the biosynthesis of tropane alkaloids.

Acknowledgement: Authors wish to thanks Government Malaysia for funding the research under Fundamental grant.

References

Aziz, A. (2001). Hairy root cultures of some medicinal plants. J. Tropic. Medic. Plants. 2: 47-56.

Aziz, A., Marziah, M., Muse, R. and Halimi, M. S. (2002). Establishment and characterization of *Datura metel* transformed root cultures. Asia Pacific J. Mol. Biol. Biotechnol. 10(1): 59-68.

Baiza, A. M., Quiroz-Moreno, A., Ruiz, J. A. and Loyola-Vargas, V. M. (1999). Genetic stability of hairy root cultures of *Datura stramonium*. Plant Cell, Tiss. Org. Cult. 59: 9-17.

Berkov, S., Zayed, R. and Doncheva, T. (2006). Alkaloid patterns in some varieties of *Datura stramonium*. Fitoterapi. 77: 179-182.

Christen, P., Aoki, T. and Shimomura, K. (1992). Characteristics of growth and tropane alkaloid production in *Hyoscyamus* albus hairy roots transformed with *Agrobacterium rhizogenes* A4. Plant Cell Rep. 11: 597-600.

Endo, T and yamada, Y. (1985). Alkaloid production in root and shoot cultures of Duboisia. Phytochemistry. 24(6): 1233-1236.

Fliniaux, M. A., Manceau, F. and Jacquin-Dubreuil, A. (1993). Simultaneous analysis of l-hyoscyamine, l-scopolamine and dl-tropic acid in plant by reversed-phase high performance liquid chromatography. J. Chromatography. 644: 193-197.

Gamborg, O. L., Miller, R. A. and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.

Granicher, F., Christen, P. and Kapetanidis, I. (1995). Production of valepotriates by hairy root cultures of *Centranthus rubber* DC. Plant Cell Rep. 14; 294-298.

Hashimoto, T. and Yamada, Y. (1987). Purification and characterization of hyoscyamine 6β-hydroxylase from root cultures of *Hyoscyamus niger* L. Hydroxylase and epoxidase activities in the enzyme preparation. Eur. J. Biochem. 164: 277-285.

Kanagae, T., Kajiya, H., Amano, Y., Hashimoto, T. and Yamada, Y. (1994). Species dependent expression of the hyoscyamine-6-hydroxylase gene in the pericycle. Plant Physiol. 105: 483-490.

Kang, S. M., Jung, H. Y., Kang, Y. M., Yun, D. J., Bahk, J. D., Yang, J. K. And Choi, M. S. (2004). Effects of methyl jasmonate and salicylic acid on the production of tropane alkloids and the expression of PMT and H6H in adventitious root cultures of Scopolia parviflora. Plant Science. 166: 745-751.

Maldonado-Mendoza, I. E., Ayora-Talavera, T. and Loyola-Vargas, V. M. (1993). Establishment of hairy root cultures of *Datura stramonium*. Characterization and stability of tropane alkaloid production during long periods of subculturing. Plant Cell Tiss. Org Cult. 33: 321-329.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15: 473-478.

Nussbaumer, P., Kapetanidis, I. and Christen, P. (1998). Hairy roots of *Datura candida X D. aurea*: Effect of culture medium composition on growth and alkaloid biosynthesis. Plant cell Rep. 17: 405-409.

Rothe, G. and Dräger, B. (2002). Tropane alkaloids-metabolic response to carbohydrate signal in root cultures of *Atropa belladonna*. Plant Science. 163 (5): 979-985.

Sato, K., Yamazaki, T. and Okuyama, E. (1991). Anthraquinone production by transformed root cultures of *Rubia tinctorum* influence of phytohormone and sucrose concentration Phytochemistry. 30: 1507-1509.

Sauerwein, M. and Shimomura, K. (1991). Alkaloids production in hairy roots of *Hyoscyamus albus* transformed with *Agrobacterium rhizogenes*. Phytochemistry. 30: 3277-3280.

Strauss, A. (1989). In Y.P.S Bajaj (ed.) Biotechnology in Agriculture and Forestry vol. 7. Springer Berlin. Pp286-314.

Vanhala, L., Eeva, M., Lapinjoki, S., Hiltunen, R. and Oksman-Caldentey, K.M. (1998). Effect of growth regulators on transformed root cultures of Hysocyamus muticus. J. Plant Physiol. 153: 475-478.

White, P. (1934). Potentially unlimited growth of excise tomato root tips in a liquid medium. Plant Physiol. 9: 585-600.

Yamada, Y. and Tabata, M. (1997). Plant biotechnology of tropane alkaloids. Plant Biotechnol. 14(1): 1-10.

Yeoman, M. M. and Yeoman, c. L. (1996). Manipulating secondary metabolism in cultured plant cells. New Phytol. 134: 553-569.

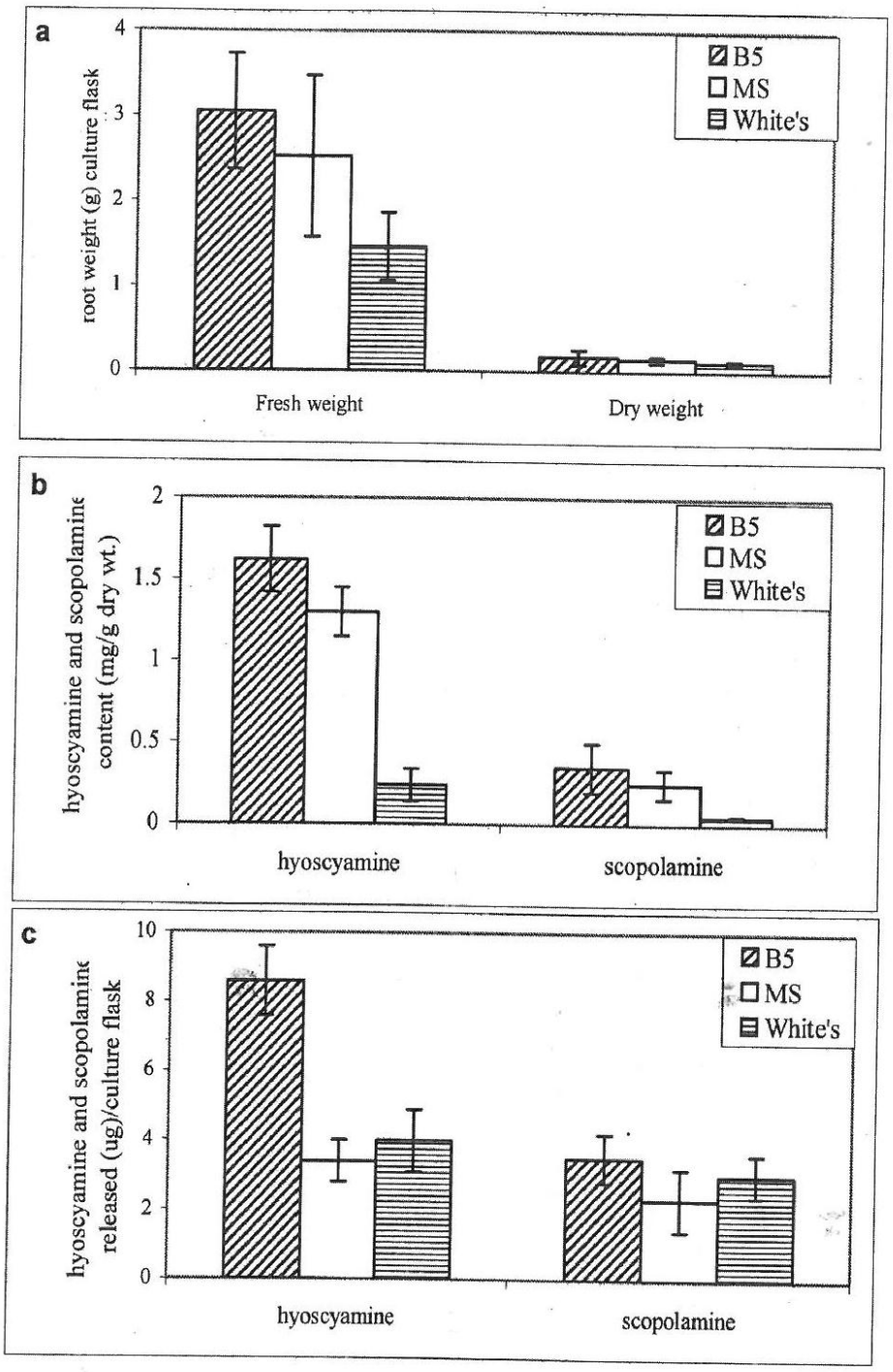

Figure 1: Effect of B5, MS and White's media on *Datura metel* transformed root cultures: root growth (a), hyoscyamine and scopolamine content in root (b), hyoscyamine and scopolamine content that was released into the media (c). Bar indicated standard error (n = 5).

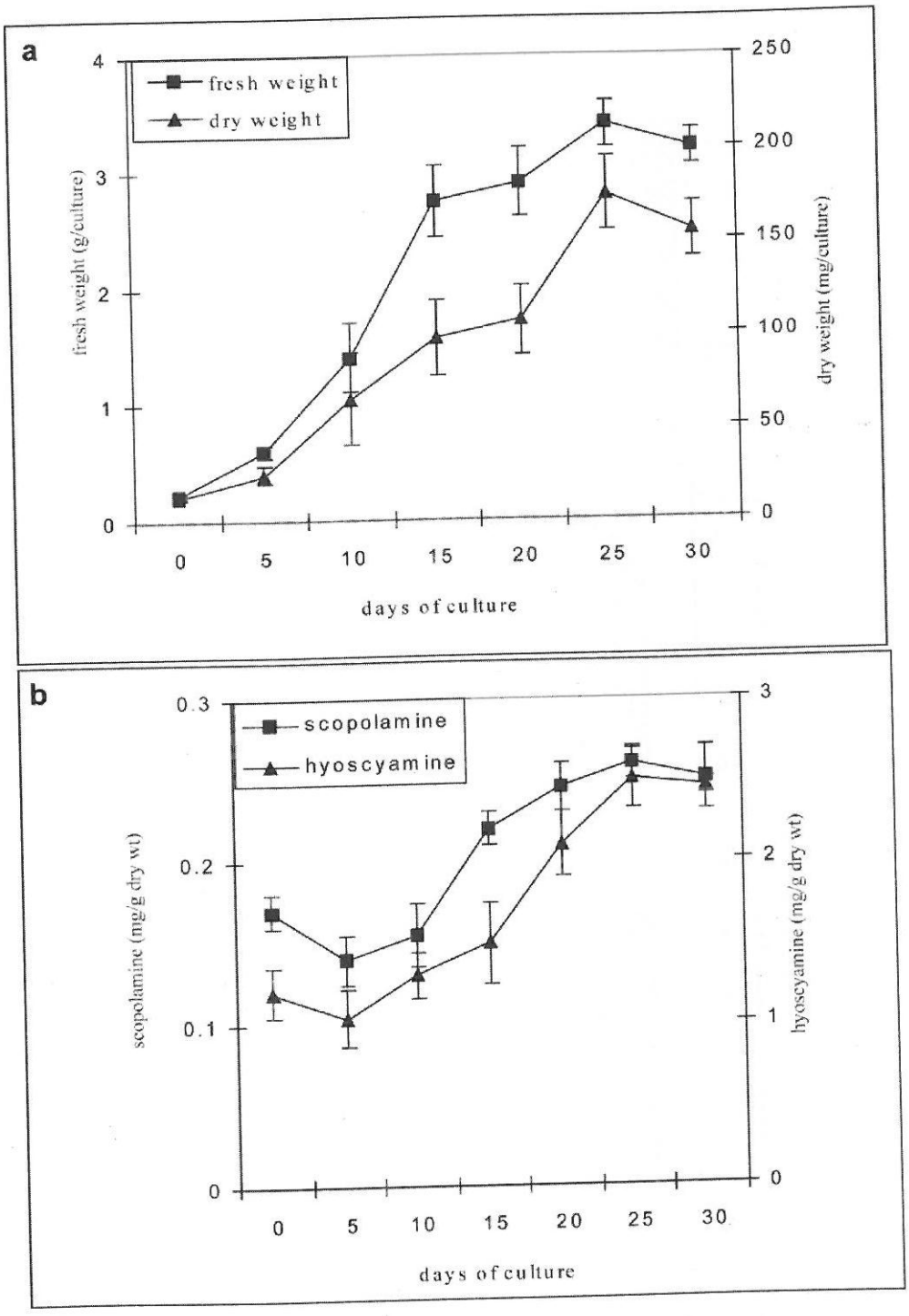

Figure 2: Root growth (a), hyoscyamine and scopolamine content (b) of *Datura metel* transformed roots cultured in Gamborg's B5 medium at different days of culture. Bar indicated standard error (n = 5).

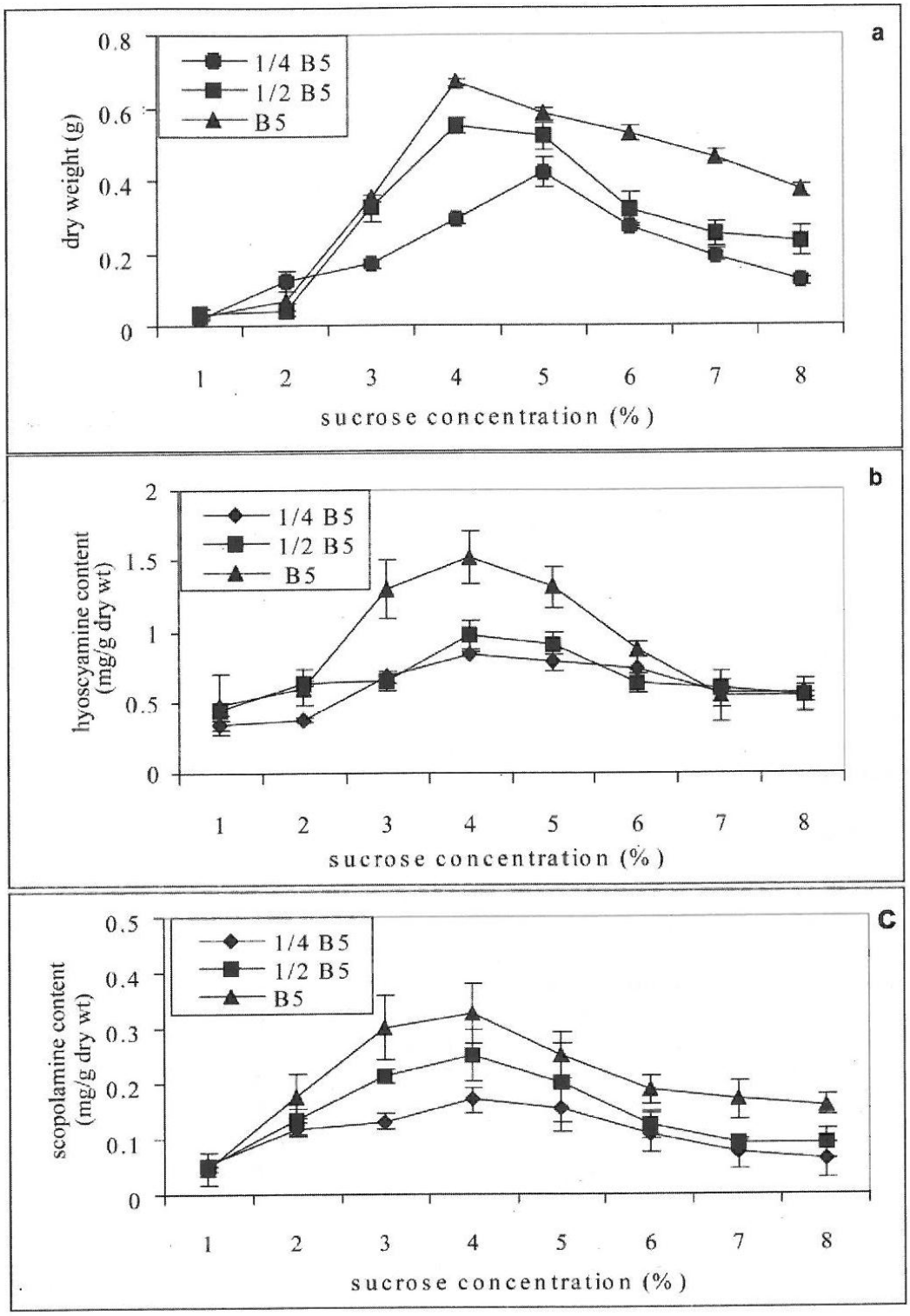

Figure 3: The effect of Gamborg's B5 medium and sucrose concentration on root dry weight (a), hyoscyamine (b) and scopolamine content (c) of *Datura metel* transformed root cultures. Bar indicated standard error (n = 5).