CHAPTER 4

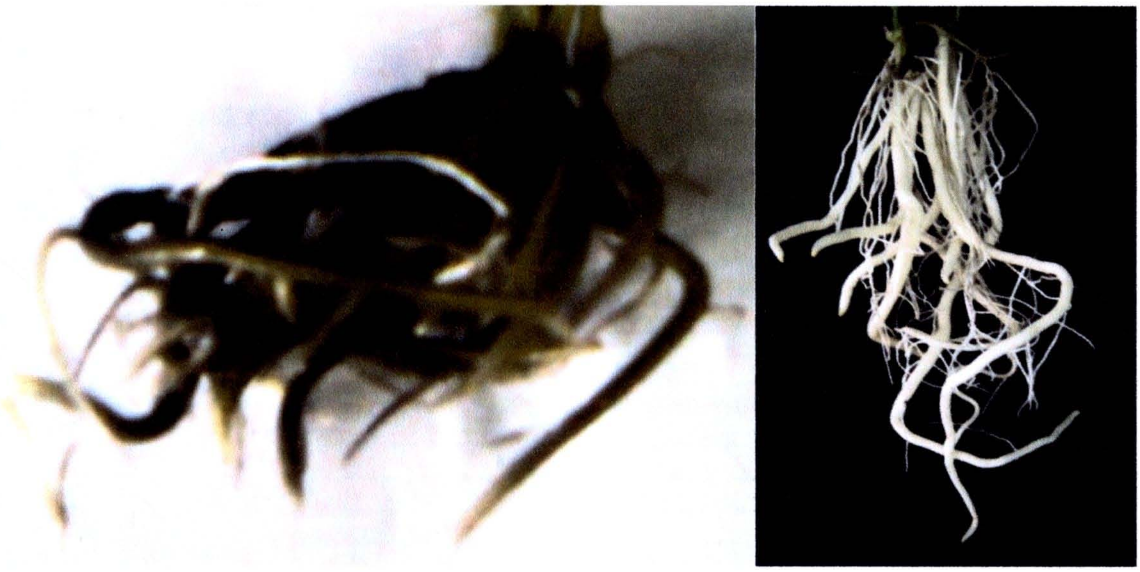
RESULTS AND DISCUSSION

4.1 Secondary compound production from root cultures of *Stemona curtisii*

4.1.1 Time profile study of alkaloid formation in root culture

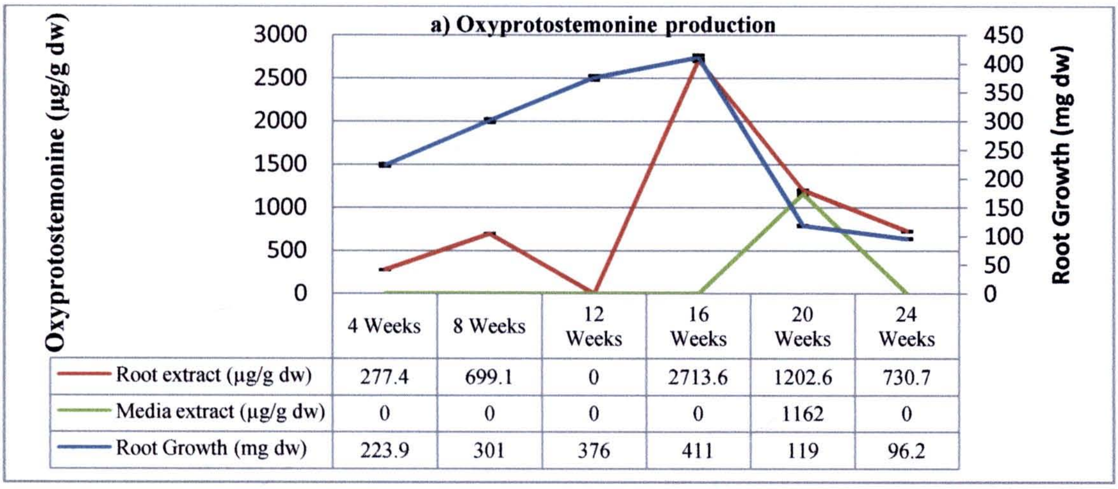
The morphology of roots grown in semisolid medium are shown in Figure 4.1a. They formed tuberous roots similar to the roots of wild plants (Figure 4.1b). The growth and alkaloid production pattern for alkaloid accumulation of *S. curtisii* root in semisolid medium is shown in Figure 4.2 and Table 4.1. The linear growth phase was between 4th-16th week. The maximum root dry weight (411±4.7 mg dw) (Table 4.1) was obtained at the end of the linear growth phase at week 16. The alkaloids accumulation in cultured root were showed the highest stemocurtisinol content (366±42 μg/g dw, 50% higher than that of intact root) was detected at week 8, at the same time oxyprotostemonine was produced at 699±4.7 μg/g dw, higher than that of stemocurtisinol content and then at 12th week the highest stemocurtisine content (24.6±0.3 μg/g dw) was obtained but oxyprtostemonine was not detected. The results indicated that oxyprotostemonine was decreased when stemocurtisinol and stemocurtisine were produced. It was possible that oxyprotostemonine was converted to stemocurtisinol (at 8th week) and stemocurtisine (at 12th week). While biosynthesis studies on *Stemona* alkaloids have not been reported, a proposed biosynthesis leading to the pyrrolo[1,2-*a*]azepine *Stemona* alkaloids has been made by Seger (2004). A-ring expansion of the pyrrolidine ring (A-ring) of oxyprotostemonine to piperidine ring was proposed to account for the biosynthesis of the pyrrolo[1,2-*a*]azepine *Stemona* alkaloids. While their biosynthetic proposal seems reasonable and give stemocurtisine (Seger *et al.*, 2004). Contrary, at 16th week the maximum of oxyprotostemonine accumulation (2,713.6±18.1 μg/g dw, 5 folds higher than of intact root) was observed when stemocurtisinol and stemocurtisine production were decreased. After the linear phase, root dry weight showed a steady decline because of the limitation of nutrients in the medium. Therefore at the 20th week, roots were died and released oxyprotostemonine, stemocurtisine and stemocurtisinol into the medium at 1,162±13.4, 11.4±0.1 and 17.5±0.2 μg/g dw, respectively. The results indicated that oxyprotostemonine, stemocurtisine and stemocurtisinol were produced in the cultured roots before release into the medium. Similarly, production of the tropane alkaloids atropine and scopolamine by *Atropa baetica* hairy roots cultured in half- and full-strength MS medium was high and also released into both liquid media. The highest tropane alkaloids present both in hairy roots and liquid half MS medium (Zárate, 1998). Many plant root cultures have been reported that a significant amount of product is released into the medium. For examples, the release of 245 mg/L scopolamine into the medium by *Duboisia Leichhardtii* hairy root cultures in modified Heller's medium was observed after 6 weeks (Muranaka *et al.* 1992) and the secretion of stemofoline and 16,17-didehydro-16(E)-stemofoline into the medium by *in vitro* plantlet of *Stemona collinsae* Craib (Burikam *et al.*, 2005). Many plant secondary compounds by cell and organ cultures were produced and accumulated intracellular. However, it may be possible to produce much higher level of product would be secreted into the medium. This is because the product intracellularly accumulated sometimes inhibits plant growth by regulation many mechanisms such as product inhibition and repression (Misawa, 1994).

This study clearly indicated that alkaloid profiles from *in vitro* root cultures of *S. curtisii* are variable to root age or cultures time and showed oxyprotostemonine was dominant alkaloid after cultured for 16 weeks. Similarly to this plant growing naturally also showed variable of alkaloid profiles depending on root age such as, previously research of Mungkornasawakul (2004) showed stemocurtisine as the major alkaloid from root extracts of this *Stemona* species growing naturally in the northern part of Trang Province in Thailand. Similar to Schinnerl (2007) isolated the alkaloid components from root extract of two different *S. curtisii* plant specimens from Chumphon. It was found that one plant sample showed stemocurtisine and stemocurtisinol as the predominant alkaloid but the other showed oxystemokerrin and its *N*-oxide as the major components and stemocurtisine and stemocurtisinol as more minor components. Furthermore, root extract of this plant growing in Krabi, Satun and Narathiwat showed that stemofoline was found as the major alkaloid component.

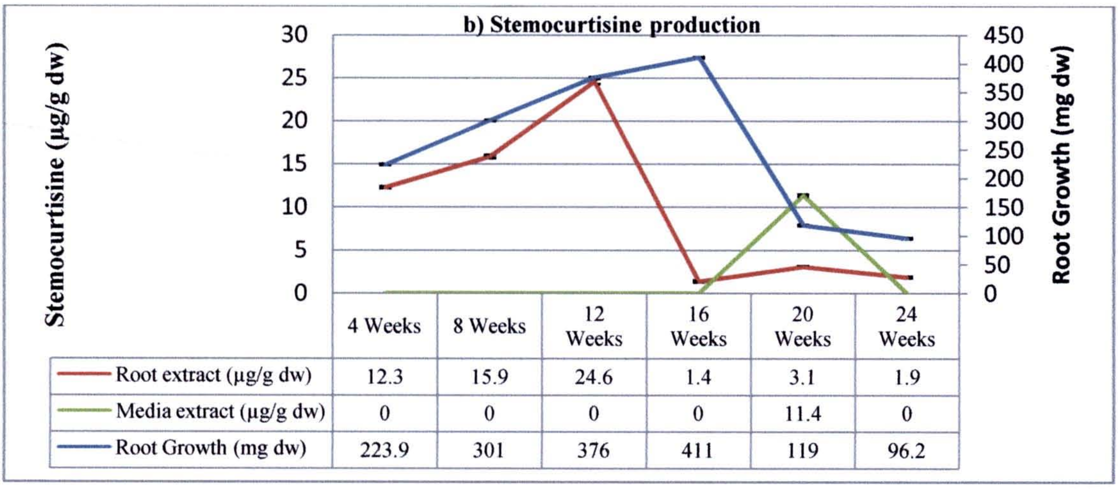


a) b)

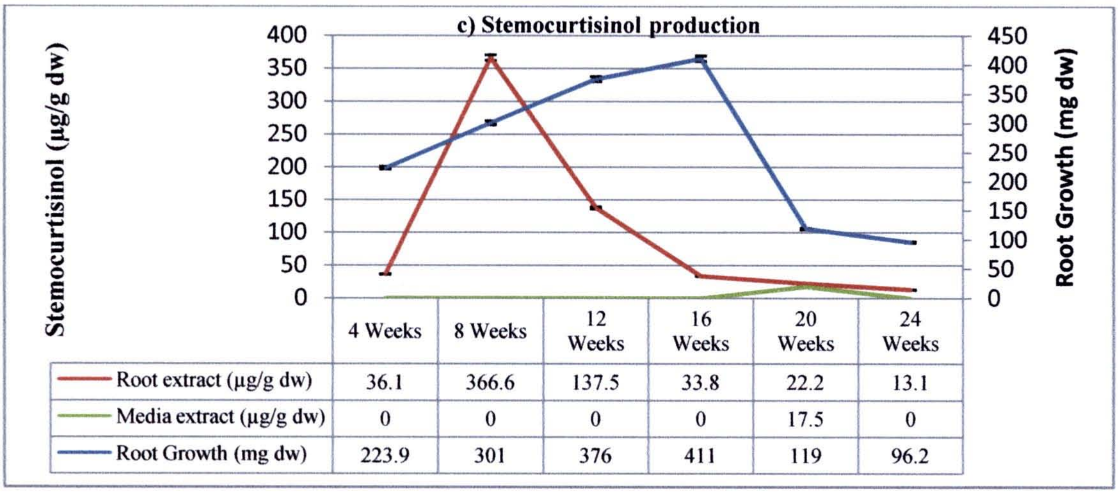
Figure 4.1 Characteristics of root cultures in semisolid cultures medium for 16 weeks a) and wild root b).



	4 Weeks	8 Weeks	12 Weeks	16 Weeks	20 Weeks	24 Weeks
Root extract (µg/g dw)	277.4	699.1	0	2713.6	1202.6	730.7
Media extract (µg/g dw)	0	0	0	0	1162	0
Root Growth (mg dw)	223.9	301	376	411	119	96.2



	4 Weeks	8 Weeks	12 Weeks	16 Weeks	20 Weeks	24 Weeks
Root extract (µg/g dw)	12.3	15.9	24.6	1.4	3.1	1.9
Media extract (µg/g dw)	0	0	0	0	11.4	0
Root Growth (mg dw)	223.9	301	376	411	119	96.2



	4 Weeks	8 Weeks	12 Weeks	16 Weeks	20 Weeks	24 Weeks
Root extract (µg/g dw)	36.1	366.6	137.5	33.8	22.2	13.1
Media extract (µg/g dw)	0	0	0	0	17.5	0
Root Growth (mg dw)	223.9	301	376	411	119	96.2

Figure 4.2 Root growth and alkaloid content of root cultures in semisolid medium. a) oxyprotostemonine production, b) stemocurtisine production and c) stemocurtisinol production.

Table 4.1 Root growth and alkaloids production from root cultures in semisolid culture medium.

Week	Root Growth (mg dw)	Alkaloid content								
		Oxyprotostemonine (µg/g dw)			Stemocurtisine (µg/g dw)			Stemocurtisinol (µg/g dw)		
		Root extract	Media extract	Total	Root extract	Media extract	Total	Root extract	Media extract	Total
4	223.9 ± 2.6^{d}	277.4 ± 1.8^{d}	0.00	277.4 ± 1.8^{d}	12.3 ± 0.1^{c}	0.00	12.3 ± 0.1^{d}	36.1 ± 0.4^{c}	0.00	36.1 ± 0.4^{c}
8	301.0 ± 3.5^{c}	699.1 ± 4.7^{c}	0.00	699.1 ± 4.7^{c}	15.9 ± 0.2^{b}	0.00	15.9 ± 0.2^{c}	366.6 ± 4.2^{a}	0.00	366.6 ± 4.2^{a}
12	376.0 ± 4.3^{b}	0.00	0.00	0.00	24.6 ± 0.3^{a}	0.00	24.6 ± 0.3^{a}	137.5 ± 1.6^{b}	0.00	137.5 ± 1.6^{b}
16	411.0 ± 4.7^{a}	$2,713.6\pm18.1^{a}$	0.00	$2,713.6\pm18.1^{a}$	1.4 ± 0.1^{e}	0.00	1.4 ± 0.1^{e}	33.8 ± 0.4^{c}	0.00	33.8 ± 0.4^{c}
20	119.0 ± 1.4^{e}	$1,202.6\pm8^{b}$	$1,162\pm13.4^{a}$	$2,364.6\pm20.8^{b}$	3.1 ± 0.1^{d}	11.4 ± 0.1^{a}	19.9 ± 0.7^{b}	22.2 ± 0.2^{d}	17.5 ± 0.2^{a}	39.8 ± 0.4^{c}
24	96.2 ± 1.1^{f}	730.7 ± 4.9^{c}	0.00	730.7 ± 4.9^{c}	1.9 ± 0.1^{e}	0.00	1.9 ± 0.1^{e}	13.1 ± 0.1^{e}	0.00	13.1 ± 0.1^{d}

Values showed the mean ± standard error followed by similar letters in a column do not differ significantly at $p<0.05$

4.1.2. Effects of elicitors on growth and *Stemona* alkaloids production in root cultures

The effects of salicylic acid on root growth and alkaloids production

A comparison was made between cultures treated with SA and controlled cultures of the root growth and oxyprotostemonine, stemocurtisine and stemocurtisinol production. SA was added on the first day of the culture period and the effects were observed at 16th week (4 months). Figure 4.3 shows the effects of SA on root growth. In this experiment, salicylic acid had a negative effect on root growth. All concentrations of SA slightly decreased root growth compared to the control (Figure 4.3) and stimulation root browning (Figure 4.20).

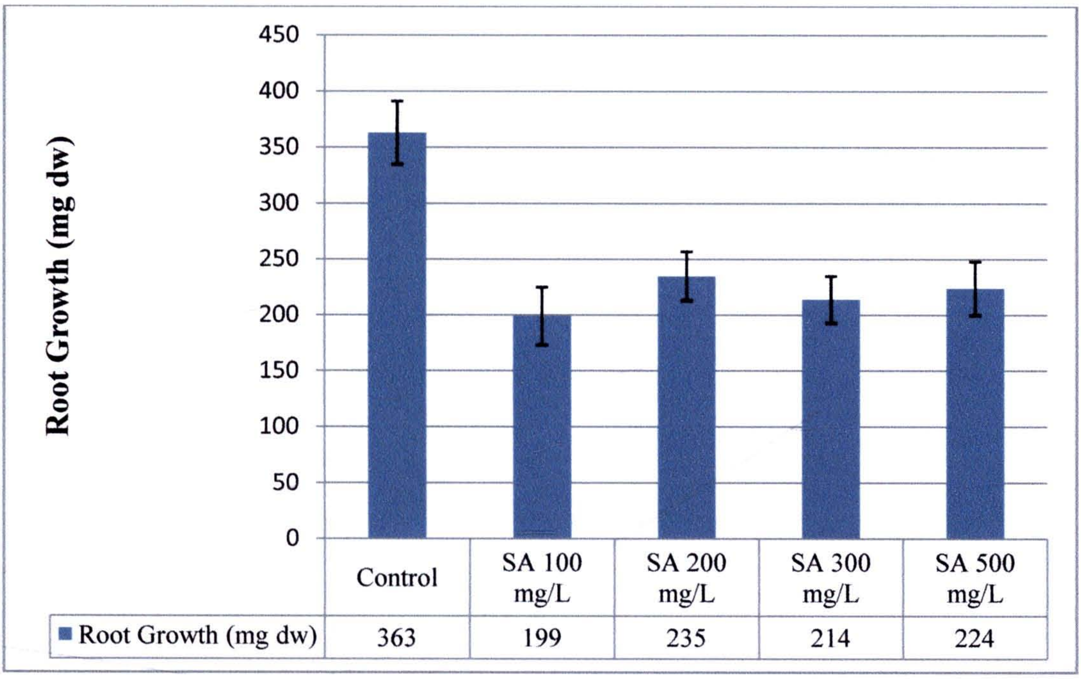
Figure 4.4 and Table 4.2 show the content of oxyprotostemonine in the root and media extracts after adding various concentrations of SA for 16 weeks. The highest significant increase of total oxyprotostemonine content (7,192±138.2 μg/g dw, 2 folds higher than that of the control and 13 folds than that of the intact roots) was observed in cultures treated with 500 mg/L SA. Increasing the SA concentration resulted in decreasing oxyprotostemonine accumulation in the root, whereas in the media it had a positive effect on stimulating its secretion which enhanced oxyprotostemonine accumulation in the media. The positive responses of the elicited cultured roots are possibly due to activation of plant defense responses (Shah *et al.*, 1999). SA has been reported to be an elicitor that can stimulate metabolite synthesis, causing the enhancement of alkaloids in *Catharanthus roseus* (Aerts *et al.*, 1996). SA can also enhance the production of some secondary metabolites in plant tissues and cell cultures. For example, treatment of *Hyoscyamus muticus* suspension cultures to 40 mM SA increased lubimin production by 50% while in transformed root cultures of the same species the addition of 4 mM SA increase solavetivone production by 48% (Mehmetoglu and Curtis, 1997). Furthermore, adding 1 mM SA to *C. roseus* callus cultures increased total alkaloid production by 5 times (Gergorio and Victor, 1997). The mechanisms by which these alkaloids are released into the media are not clear. It is possible that SA induced roots death resulting in the release of these metabolites into the media. Investigations have revealed the storage of phytochemicals in vacuoles. The accumulation of indole alkaloids in the vacuoles of C. *roseus* has been attributed to an ion-trap mechanism, whereby the basic indole alkaloids are trapped in the acidic vacuole due to the alkaloids positive charge at low pH, preventing diffusion across the tonoplast (Neumann *et al.*, 1983). Studies carried out with C. *roseus* suspension cultures led to the proposal of an 'ion-trapping' mechanism of alkaloids in the vacuoles, involving their passive diffusion in the neutral state from the cytoplasm towards the vacuoles, where they would be protonated (positively charged) and maintained trapped. These studies revealed that lowering the medium pH, and thus changing the pH gradient between the cells and the medium, would induce the release of alkaloids and their entrapment as ions in the extracellular environment (Renaudin and Guern, 1990). However, a detailed study of the releasing mechanism of *S. curtisii* root cultures is required in future studies.

Effects of SA on stemocurtisine production are shown in Figure 4.5 and Table 4.2. Similar to the oxyprotostemonine production results, treatment with exogenous SA also increased the production of stemocurtisine and the release of this metabolite into the culture medium. Increasing the salicylic acid concentration resulted in a decreased stemocurtisine content in the root but increased the release of stemocurtisine into the cultured media. Treatment with 500 mg/L SA resulted in a significant increase in the total stemocurtisine content (39±0.4 µg/g dw) up to about 39 folds higher than that of the control. However, stemocurtisine production was overall 9 folds less than that found in the intact root.

Effects of SA on stemocurtisinol production were investigated and the results are shown in Figure 4.6 and Table 4.2. Similar to the stemocurtisine production results, treatment with exogenous SA had a positive effect on production of stemocurtisinol and the release of this metabolite into the culture medium. Treatment with 500 mg/L SA significantly increased the total stemocurtisinol content (197±2.1 µg/g dw) up to about 5 folds higher than that of the control. However, stemocurtisinol production was less than that found in the intact root.

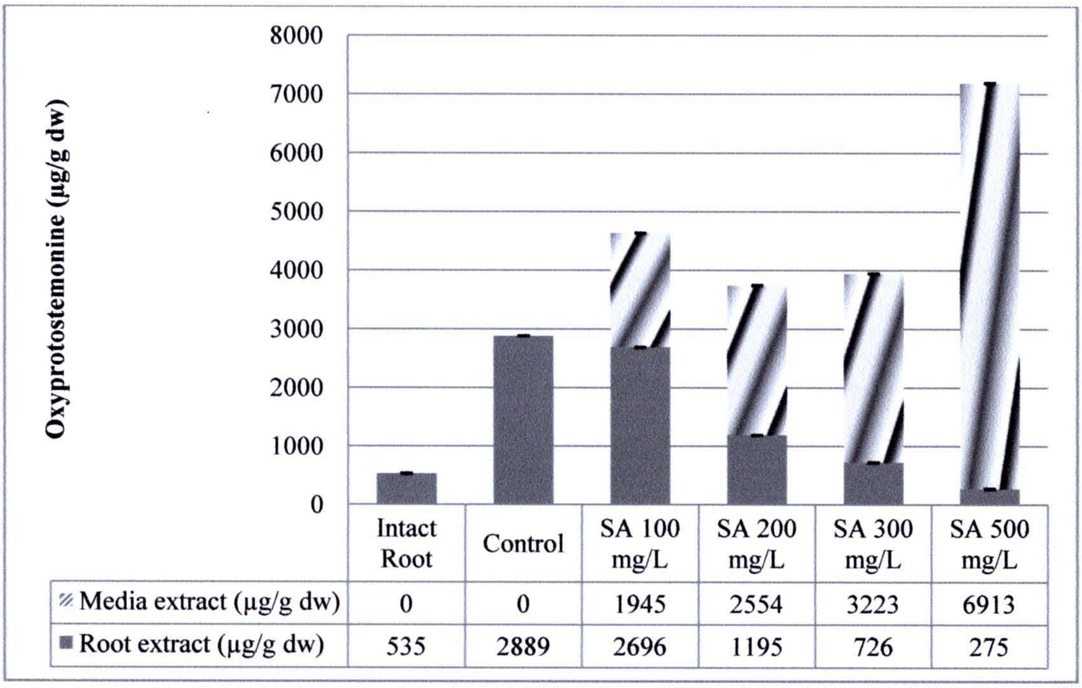
The secretion of oxyprotostemonine, stemocurtisine and stemocurtisinol into the culture media during SA treatment indicated that SA can stimulate the release of oxyprotostemonine, stemocurtisine and stemocurtisinol from *S. curtisii* roots in cultures with continued biosynthetic activity depending on the concentration of SA.

Increasing in oxyprotostemonine production with respect to SA addition could be attributed to the fact that SA act as powerful inducing signals for secondary metabolite synthesis, and plays an essential role in many plant defense reactions (Durner *et al.*, 1997). It is known to regulate the expression of various defense related genes (Malamy *et al.*, 1990), Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. SA is also regarded as a signal molecule playing an important role in Systemic Acquired Resistance (SAR) and inhibitor of ethylene biosynthesis (Dong, 1998). Therefore, the possibilities exist that SA could be acting through the inhibition of ethylene biosynthesis, a phytohormone which is active in plant defense mechanism (O'Donnell *et al.*, 1996). Oxyprotostemonine was the defense chemical of plants against insects could be regulated with the presence of the signal molecule (Mungkornasawakul *et al.*, 2003).



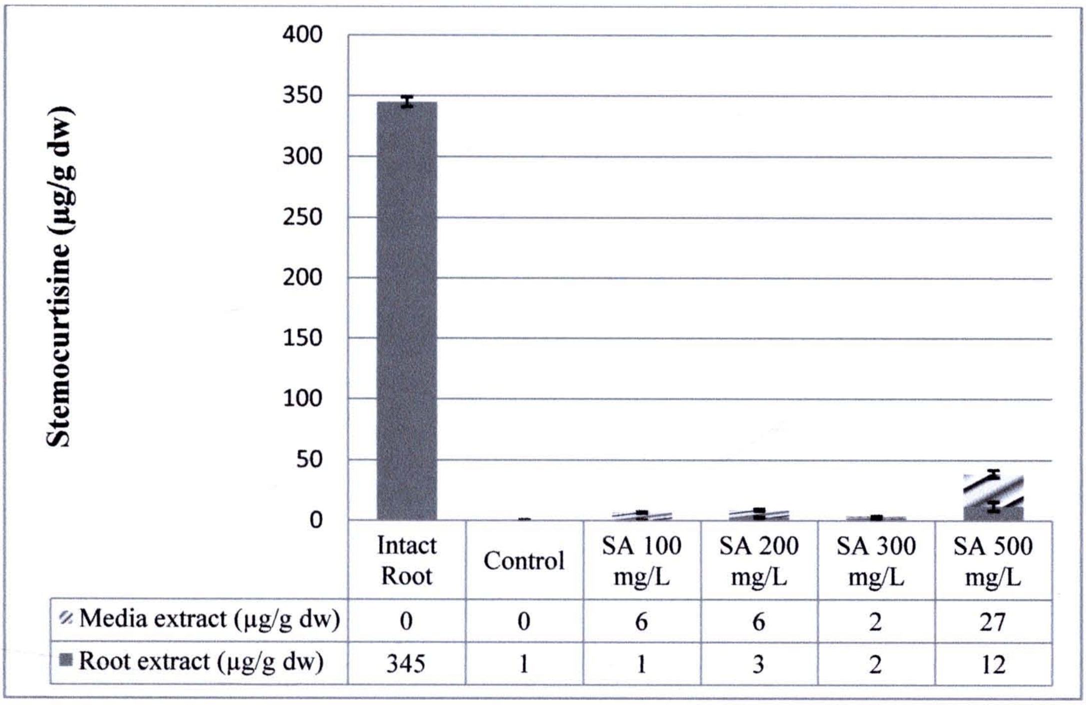
	Control	SA 100 mg/L	SA 200 mg/L	SA 300 mg/L	SA 500 mg/L
■ Root Growth (mg dw)	363	199	235	214	224

Figure 4.3 The effects of salicylic acid on root growth.



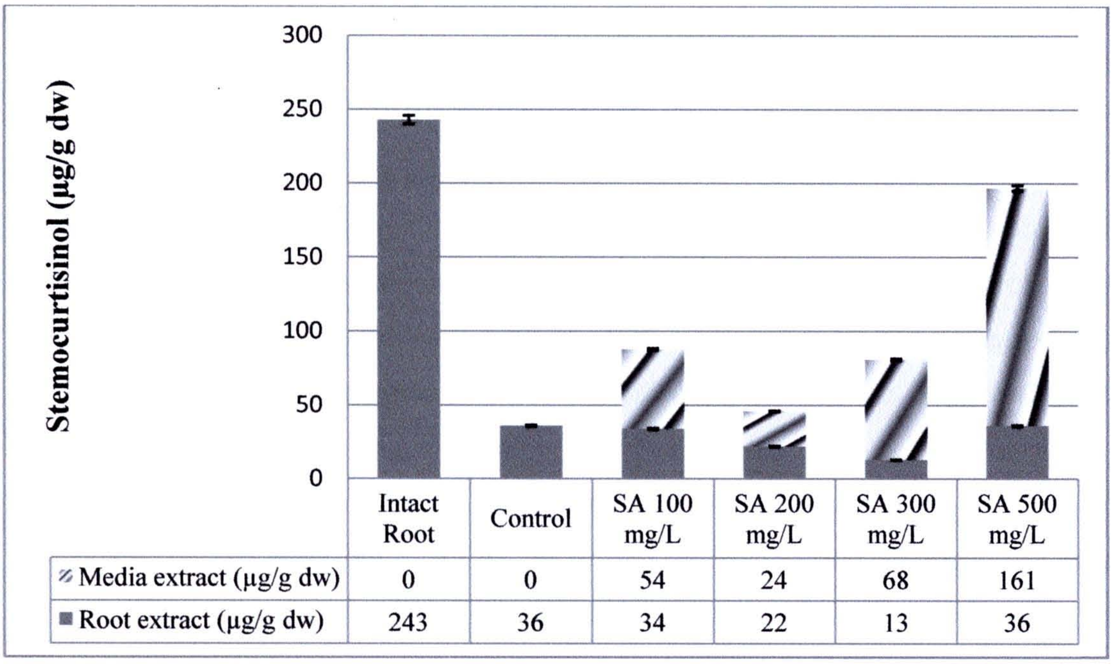
	Intact Root	Control	SA 100 mg/L	SA 200 mg/L	SA 300 mg/L	SA 500 mg/L
▨ Media extract (µg/g dw)	0	0	1945	2554	3223	6913
■ Root extract (µg/g dw)	535	2889	2696	1195	726	275

Figure 4.4 The effects of salicylic acid on oxyprotostemonine production.



	Intact Root	Control	SA 100 mg/L	SA 200 mg/L	SA 300 mg/L	SA 500 mg/L
▨ Media extract (µg/g dw)	0	0	6	6	2	27
■ Root extract (µg/g dw)	345	1	1	3	2	12

Figure 4.5 The effects of salicylic acid on stemocurtisine production.



	Intact Root	Control	SA 100 mg/L	SA 200 mg/L	SA 300 mg/L	SA 500 mg/L
▨ Media extract (µg/g dw)	0	0	54	24	68	161
■ Root extract (µg/g dw)	243	36	34	22	13	36

Figure 4.6 The effects of salicylic acid on stemocurtisinol production.

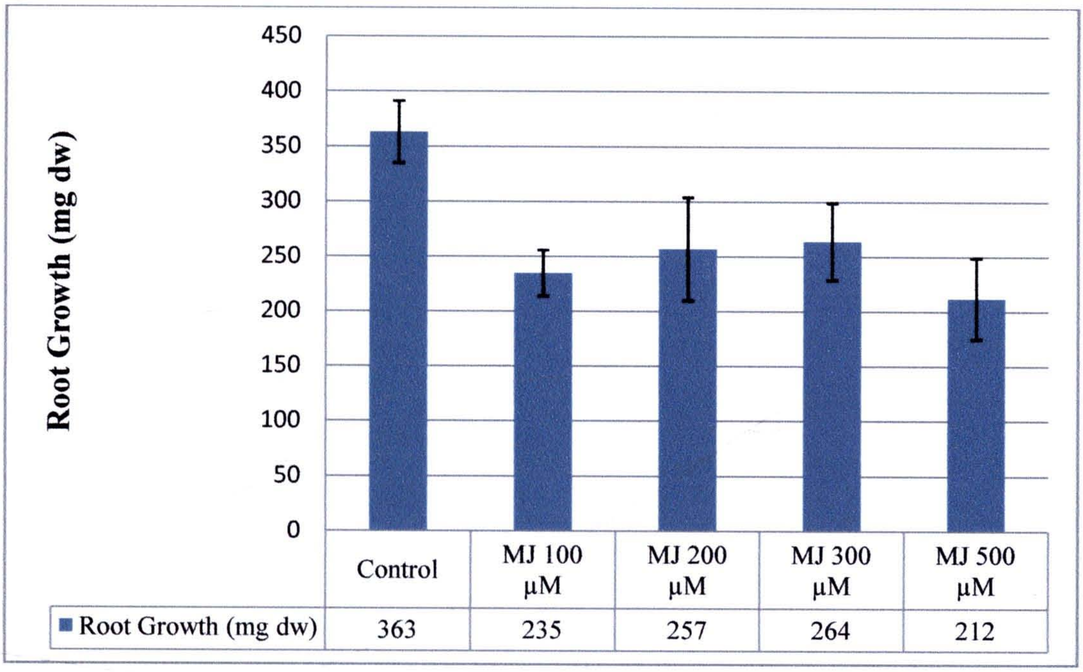
The effects of methyl jasmonate (MJ) on root growth and production of oxyprotostemonine, stemocurtisine and stemocurtisinol

Root growth was compared in cultures treated with the elicitor MJ to that of the control cultures. MJ was added on first day and its effects were observed at 16 weeks. The MJ treatment led to a repression of root growth (Figure 4.7 and Table 4.2) and induced root browning (Figure 4.20). Previous researchers have reported that 0.01mM MJ inhibited root growth in some species (Lois *et al.*, 1989) while MJ has been reported to have no effective on plant growth but inhibit the metabolic activities in soybean (Cosio *et al.*, 1990).

In this study, we analysed the accumulation of three alkaloids already identified in *S. curtisii* roots, oxyprotostemonine, stemocurtisine and stemocurtisinol. The amount of oxyprotostemonine decreased significantly in all concentrations of MJ treatments when compared to control (Figure 4.8). The highest level of total oxyprotostemonine accumulation was observed with the control at 2,927±19.2 μg/g dw (Table 4.2). Moreover oxyprotostemonine production was inhibited with 200μM MJ treatment but increases again at 300 μM MJ treatment. It is possible that at 200μM MJ, oxyprotostemonine maybe converted to stemocurtisine and stemocurtisinol because of the maximum total stemocurtisine production (25±0.2 μg/g dw, 25 folds over control) was observed on this treatment (Figure 4.9). The 100 μM MJ increased the stemocurtisinol content up to 10 folds compared with the control (Figure 4.10).

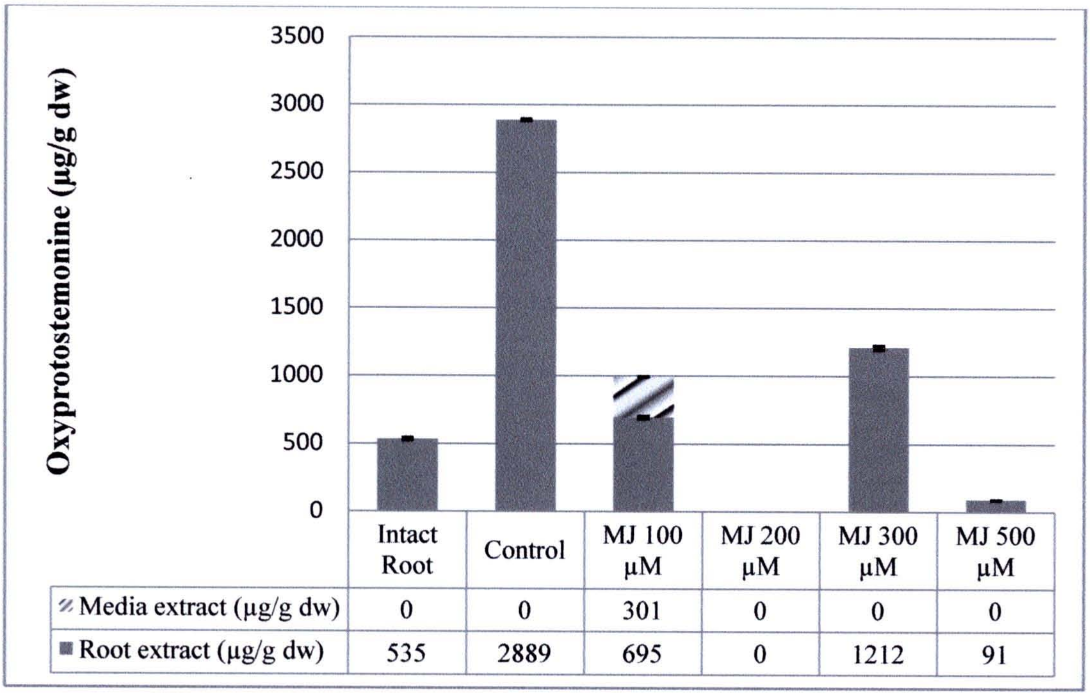
The effect of MJ on the production of stemocurtisine was maximal at 200 μM, whereas the effect of MJ on the production of stemocurtisinol was maximal at 100 μM. This result indicated that the optimal concentration of elicitor to induced plant metabolite production was dependent on the kinds of metabolite. MJ has been shown to stimulate the production of metabolites in other plant cell cultures for examples, Camptothecin production in suspension cultures of *Camptotheca acuminate* was increased 11 times by using the optimum dosing concentration of jasmonic acid which was 50 μM. The kinetics of camptothecin accumulation in response to the treatment with jasmonic acid showed that the camptothecin accumulation reached the maximum value at 4 days after jasmonic acid dosing and then a rapid decrease in camptothecin accumulation was observed (Song and Byun, 1998). Kang (2004) reported the production of tropane alkaloids in adventitious root cultures of *Scopolia parviflora*. MJ treatments increased the amounts of both scopolamine and hyoscyamine in adventitious root cultures of *Scopolia parviflora*, with growth inhibition of the roots.

MJ is known to be a key signaling compound in plants activated upon insect feeding (Walling, 2000). It is shown that MJ is also associated with the accumulation of some secondary metabolites (van der Fits and Memelink, 2000). Our experiments showed that MJ increased the content of stemocurtisinol. This corresponds with previous reports that MJ can elicit the accumulation of alkaloids (Aerts *et al*., 1994; Zabetakis *et al*., 1999), phenolics (Lee *et al*., 1997) or coumarins (Sharan *et al*., 1998) in plants. The effects of MJ on plant secondary metabolism promotion has been reported to be due to the elicitor promoting signal transducers for the production of plant secondary metabolites (Gundlach *et al*. 1992). They induce an accumulation of compounds belonging to different structural classes, including, phenolics, terpenoids, and alkaloids. A jasmonate-responsive transcription factor linking plant stress responses to changes in metabolism was isolated from *Catharanthus roseus* (van der Fits and Memelink, 2000).



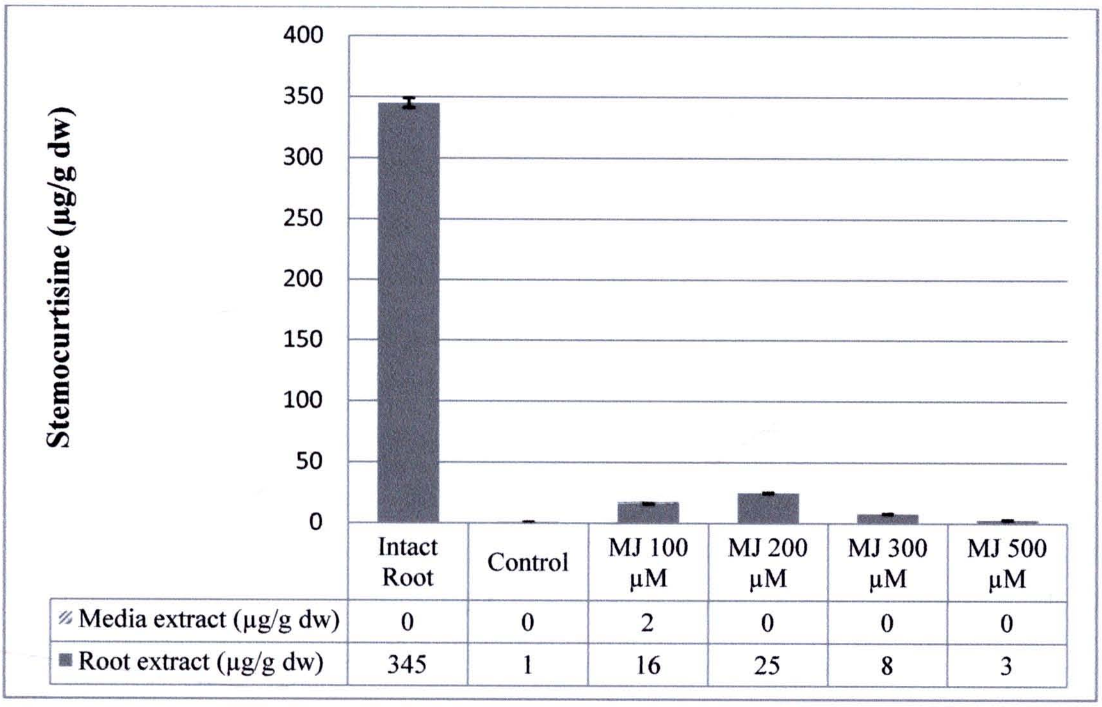
	Control	MJ 100 µM	MJ 200 µM	MJ 300 µM	MJ 500 µM
■ Root Growth (mg dw)	363	235	257	264	212

Figure 4.7 The effects of methyl jasmonate on root growth



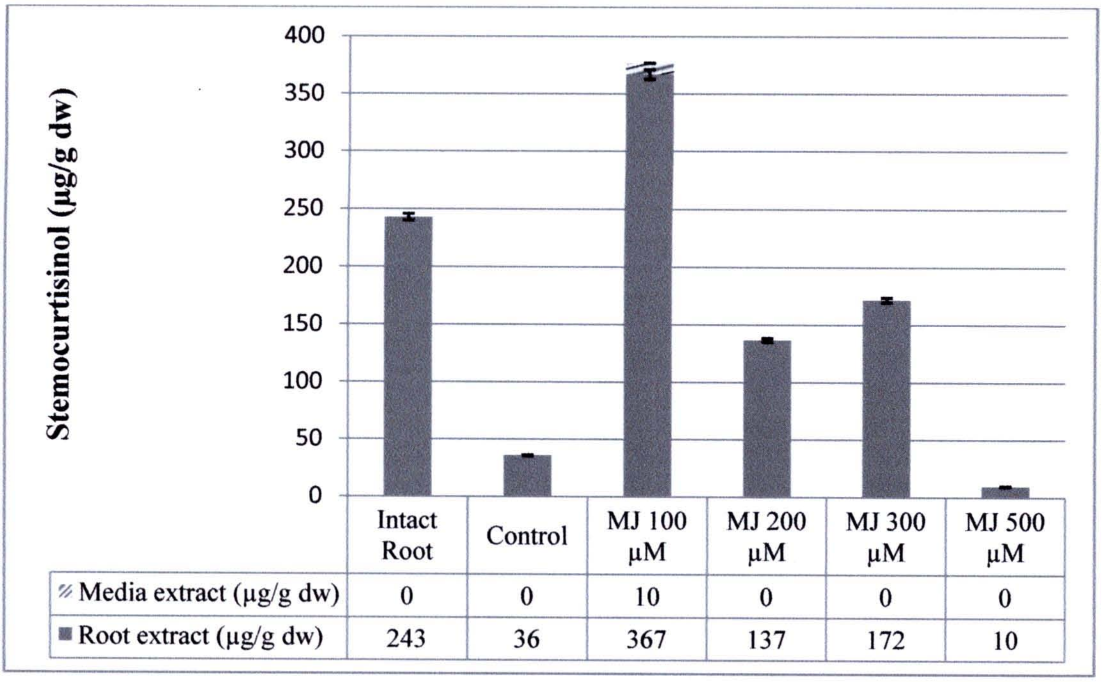
	Intact Root	Control	MJ 100 µM	MJ 200 µM	MJ 300 µM	MJ 500 µM
▨ Media extract (µg/g dw)	0	0	301	0	0	0
■ Root extract (µg/g dw)	535	2889	695	0	1212	91

Figure 4.8 The effects of methyl jasmonate on oxyprotostemonine production.



	Intact Root	Control	MJ 100 µM	MJ 200 µM	MJ 300 µM	MJ 500 µM
▨ Media extract (µg/g dw)	0	0	2	0	0	0
■ Root extract (µg/g dw)	345	1	16	25	8	3

Figure 4.9 The effects of methyl jasmonate on stemocurtisine production.



	Intact Root	Control	MJ 100 µM	MJ 200 µM	MJ 300 µM	MJ 500 µM
▨ Media extract (µg/g dw)	0	0	10	0	0	0
■ Root extract (µg/g dw)	243	36	367	137	172	10

Figure 4.10 The effects of methyl jasmonate on stemocurtisinol production.

The effects of yeast extract (YE) on root growth and production of oxyprotostemonine, stemocurtisine and stemocurtisinol

Increasing concentrations of yeast extract (0.5-5 g/L) in the medium resulted in decreasing root growth (Figure 4.11 and Table 4.2) and oxyprotostemonine accumulation (Figure 4.12). The inclusion of yeast extract at the concentration of 2 g/L promoted the production of total stemocurtisine (Figure 4.13) and stemocurtisinol (Figure 4.14). However, the relatively higher concentration of yeast extract (5 g/L) resulted in a decline of alkaloid content. The highest level of total stemocurtisine (5±0.1 μg/g dw) and stemocurtisinol (91±11.5 μg/g dw) were obtained at 2 g/L yeast extract, representing about 5 and 2.5 times more than the control, respectively. Yeast extract has been reported to slightly increase the hyoscyamine content and increase the scopolamine 2.5 times in *Datura stramonium* cultures (Zabetakis *et al.*, 1999). Yeast extract is composed of a variety of compounds, including amino acids, vitamins and minerals (Ertola and Hours, 1998) and it is also possible that elicitation effects might be due to cations like Zn, Ca and Co in the yeast extract (Ajungla *et al.*, 2009).

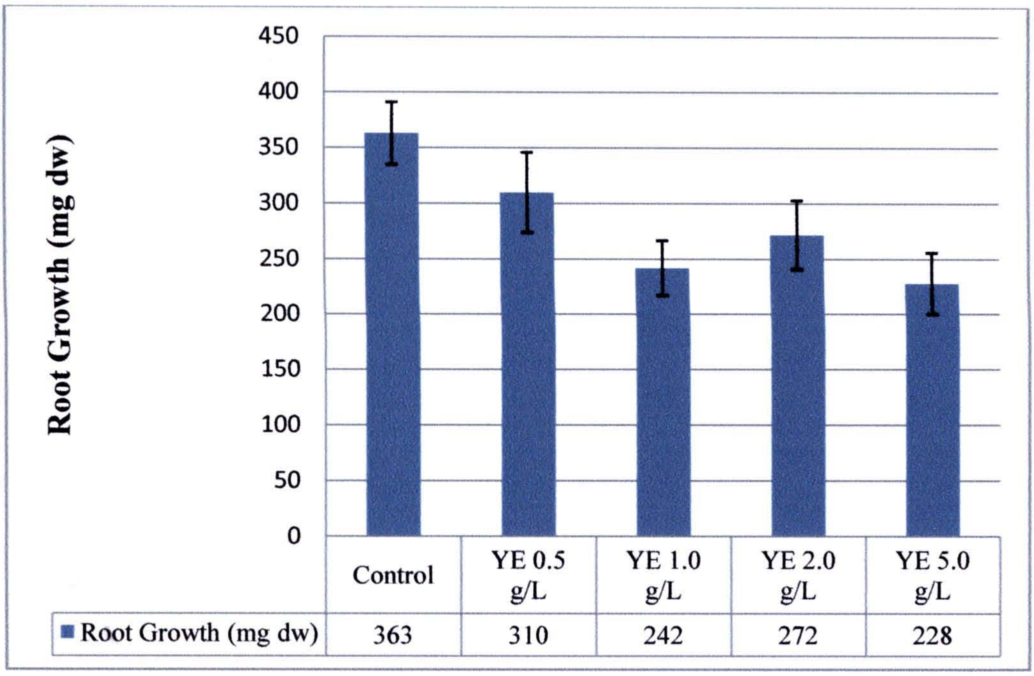
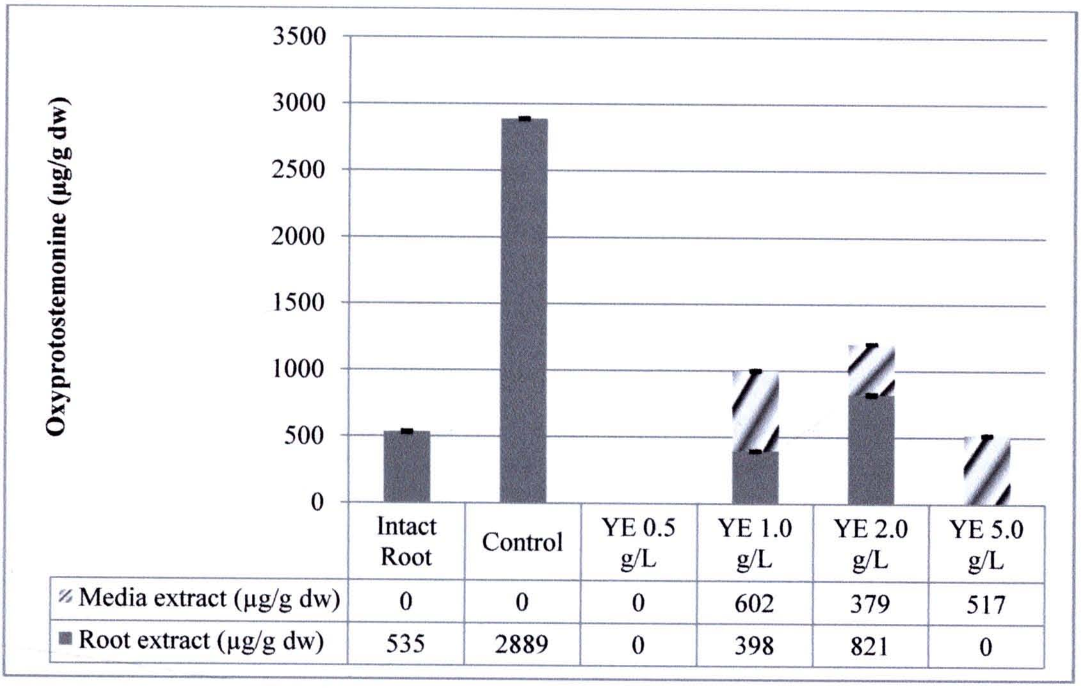
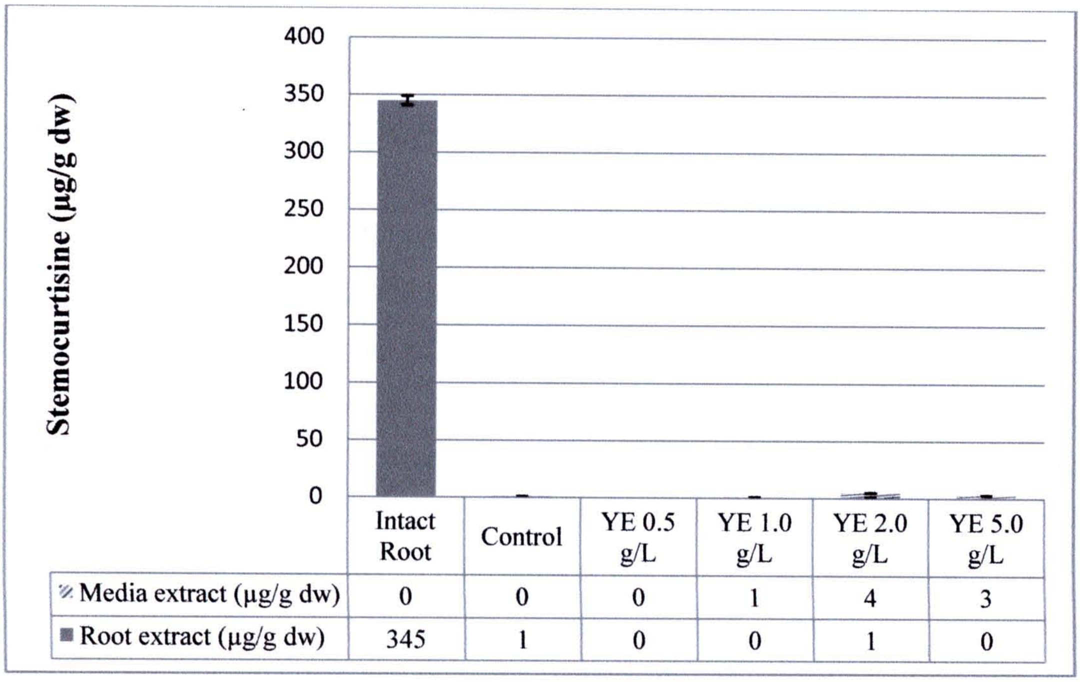


Figure 4.11 The effects of yeast extract on root growth



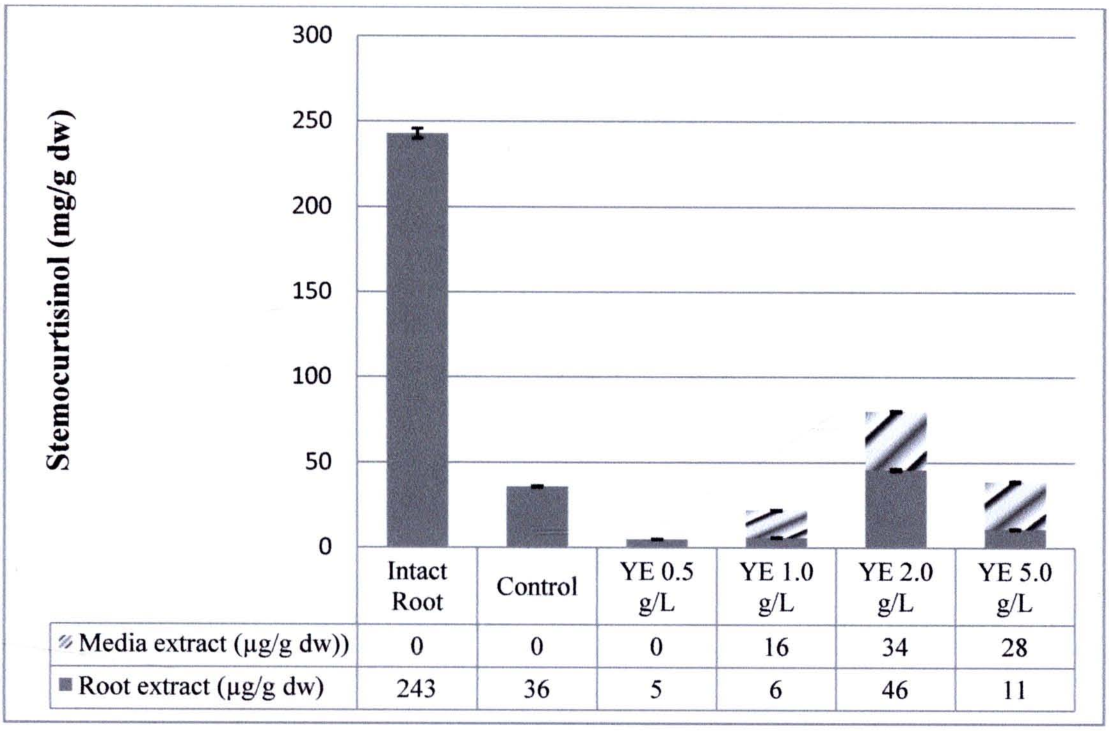
	Intact Root	Control	YE 0.5 g/L	YE 1.0 g/L	YE 2.0 g/L	YE 5.0 g/L
▨ Media extract (µg/g dw)	0	0	0	602	379	517
■ Root extract (µg/g dw)	535	2889	0	398	821	0

Figure 4.12 The effects of yeast extract on oxyprotostemonine production.



	Intact Root	Control	YE 0.5 g/L	YE 1.0 g/L	YE 2.0 g/L	YE 5.0 g/L
▨ Media extract (µg/g dw)	0	0	0	1	4	3
■ Root extract (µg/g dw)	345	1	0	0	1	0

Figure 4.13 The effects of yeast extract on stemocurtisine production.

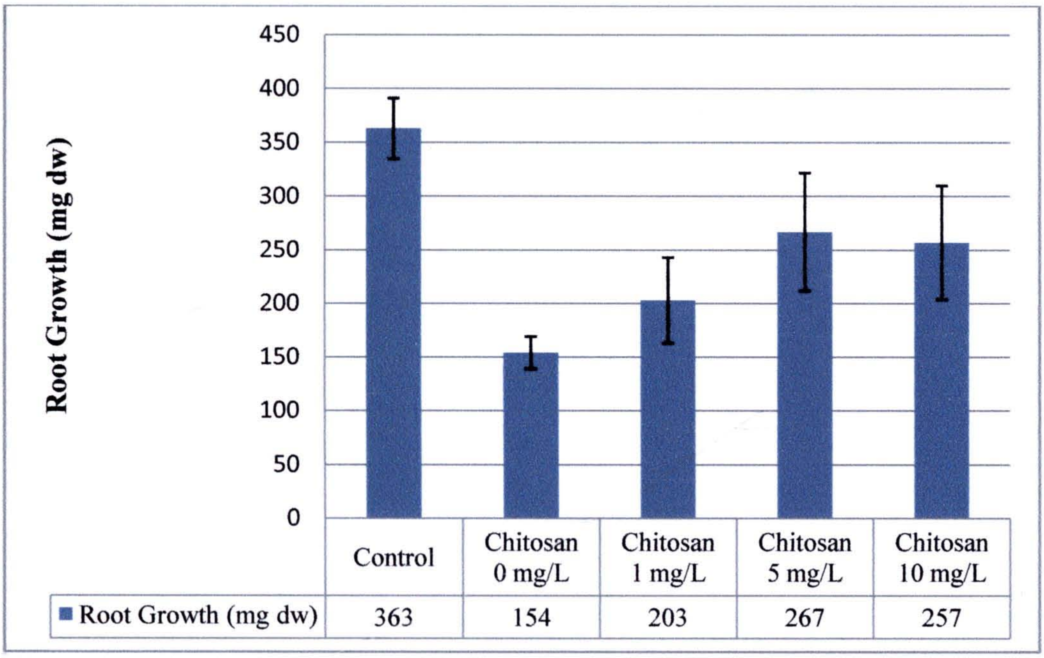


	Intact Root	Control	YE 0.5 g/L	YE 1.0 g/L	YE 2.0 g/L	YE 5.0 g/L
▨ Media extract (µg/g dw))	0	0	0	16	34	28
■ Root extract (µg/g dw)	243	36	5	6	46	11

Figure 4.14 The effects of yeast extract on stemocurtisinol production.

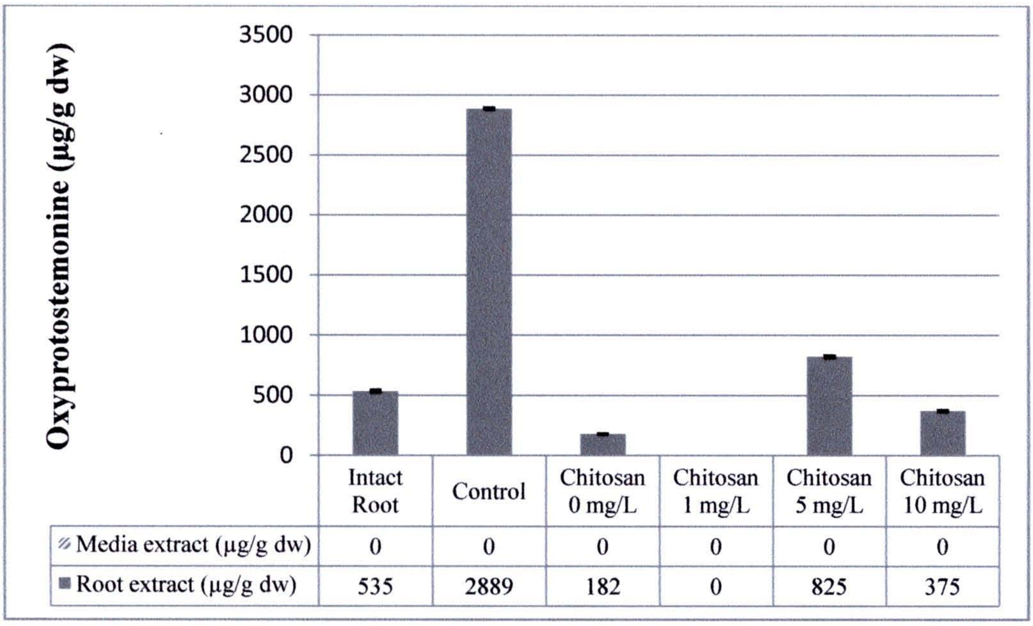
Effects of chitosan on growth and oxyprotostemonine, stemocurtisine and stemocurtisinol production

Figure 4.15 shows the effect of chitosan on root growth of *S. curtisii*. Growth was not significantly affected throughout the 16 weeks (Table 4.2). Figure 4.16, shows the effect of chitosan on oxyprotostemonine production. In this experiment, chitosan had a negative effect on oxyprotostemonine accumulation. All treatments with chitosan significantly decreased oxyprotostemonine content, much lower than that of control. On the other hand, all concentrations of chitosan showed no actual elicitor effects on stemocurtisine (Figure 4.17) and stemocurtisinol accumulation (Figure 4.18). This was probably due to low concentrations of chitosan causing cells were not elicited (Kim *et al.*, 1997).



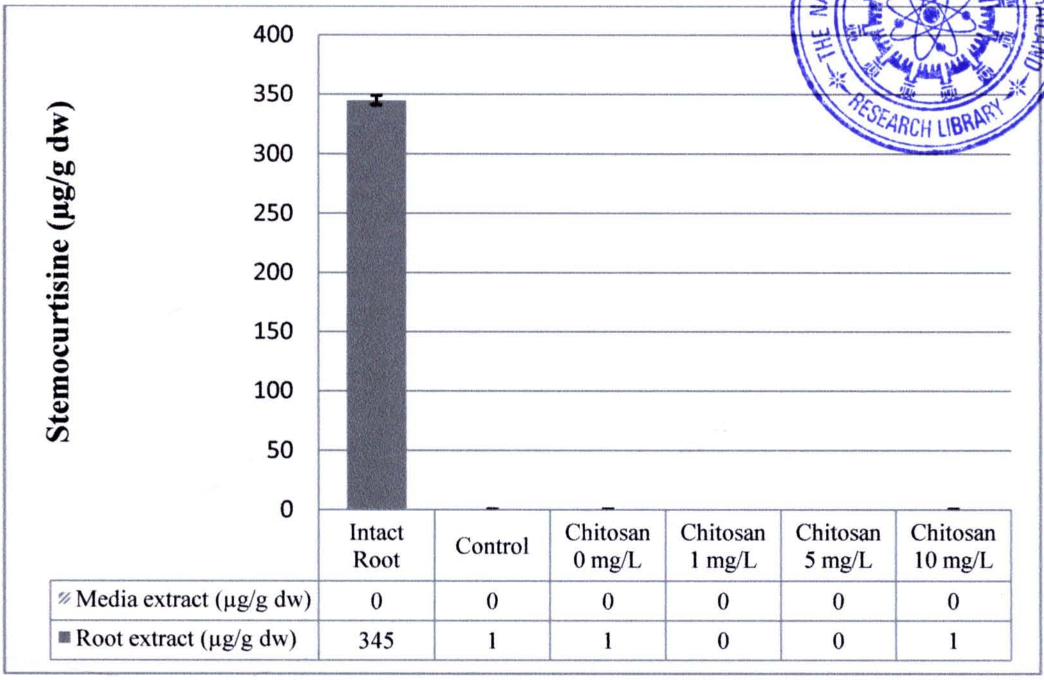
	Control	Chitosan 0 mg/L	Chitosan 1 mg/L	Chitosan 5 mg/L	Chitosan 10 mg/L
■ Root Growth (mg dw)	363	154	203	267	257

Figure 4.15 The effects of chitosan on root growth



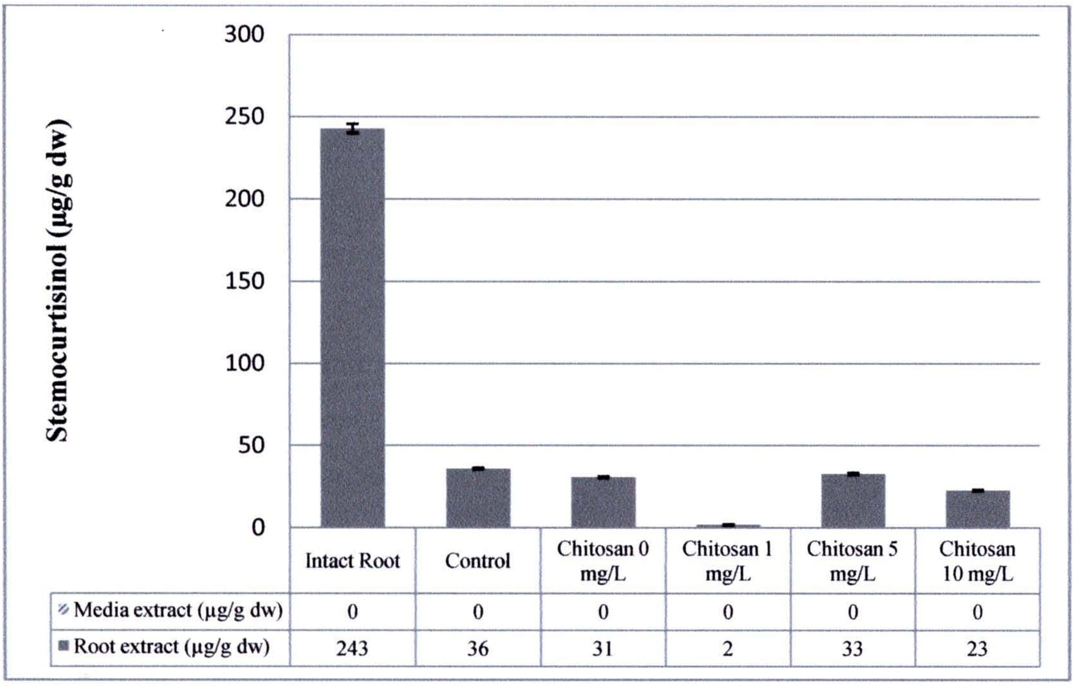
	Intact Root	Control	Chitosan 0 mg/L	Chitosan 1 mg/L	Chitosan 5 mg/L	Chitosan 10 mg/L
▨ Media extract (µg/g dw)	0	0	0	0	0	0
■ Root extract (µg/g dw)	535	2889	182	0	825	375

Figure 4.16 The effects of chitosan on oxyprotostemonine production.



	Intact Root	Control	Chitosan 0 mg/L	Chitosan 1 mg/L	Chitosan 5 mg/L	Chitosan 10 mg/L
▨ Media extract (µg/g dw)	0	0	0	0	0	0
■ Root extract (µg/g dw)	345	1	1	0	0	1

Figure 4.17 The effects of chitosan on stemocurtisine production.



	Intact Root	Control	Chitosan 0 mg/L	Chitosan 1 mg/L	Chitosan 5 mg/L	Chitosan 10 mg/L
▨ Media extract (µg/g dw)	0	0	0	0	0	0
■ Root extract (µg/g dw)	243	36	31	2	33	23

Figure 4.18 The effects of chitosan on stemocurtisinol production.

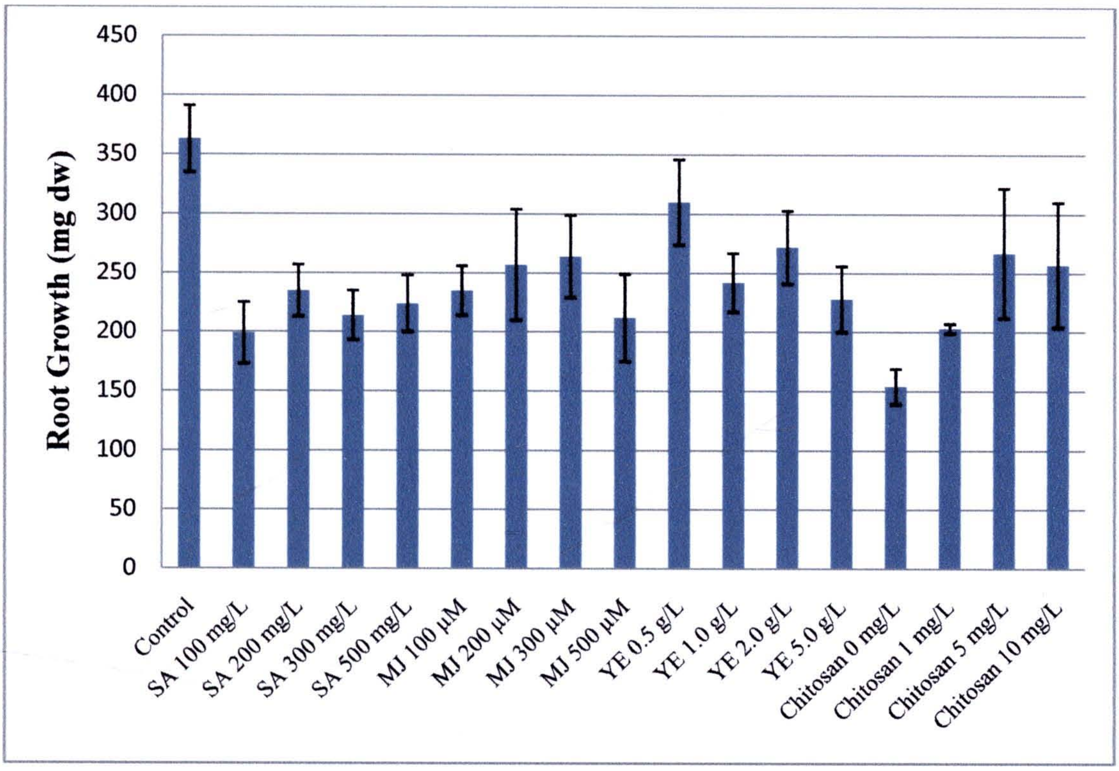


Figure 4.19 The effects of elicitors on root growth

Conclusion

Figure 4.19 and Table 4.2 demonstrate the effects of elicitors on root growth after cultured for 16 weeks. The results investigated that all of elicitor treatments decreased root growth when compared with control but non-significantly difference with the exception of 0 mg/L chitosan treatment had significantly lower root growth than that of control. The morphology of roots grown in elicitors are shown in Figure 4.20. When elicitors were used, roots were was no apparent change in color or morphology of roots treated with chitosan whereas in other elicitor treatments, light-green-colored roots turned brown and necrosis without formation of tuberous roots. Especially, root treated with SA show dark brown colored roots and also decreased root growth. Whereas increasing concentration of SA had positive effect on *Stemona* alkaloids production. It was found that 500 mg/L SA treatment induced highest of oxyprotostemonine production at 7,192±138 µg/g dw and most of this alkaloid was released into the medium.

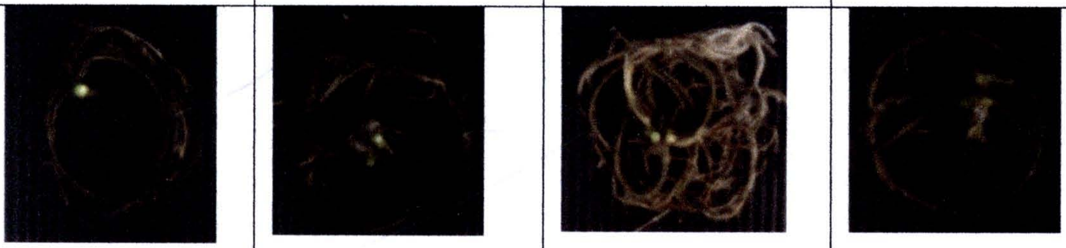
Table 4.2 Influence of elicitor on root growth and alkaloid content in root and cultures media of *S. curtisii*

Influence of elicitor on the alkaloid content in root and cultures media of *S. curtisii.*										
		Alkaloid content								
	Root Growth	Oxyprotostemonine (µg/g dw)			Stemocurtisine (µg/g dw)			Stemocurtisinol (µg/g dw)		
	(mg dw)	Root extract	Media extract	Total	Root extract	Media extract	Total	Root extract	Media extract	Total
Intact root	-	535 ± 8.66^{i}	-	535 ± 8.66^{i}	345 ± 3.89^{a}	-	345 ± 3.89^{a}	243 ± 2.80^{b}	-	243 ± 2.80^{b}
Control	363 ± 0.028^{a}	$2{,}889\pm19.2^{a}$	0	$2{,}889\pm19.2^{e}$	1 ± 0.01^{ef}	0	1 ± 0.1^{fg}	36 ± 0.41^{f}	0	36 ± 0.2^{efgh}
Salicylic acid (mg/L)										
100	199 ± 26^{ab}	$2{,}696\pm30.89^{b}$	$1{,}945\pm22^{d}$	$4{,}677\pm28.8^{b}$	1 ± 0.01^{ef}	6 ± 0.6^{b}	8 ± 0.1^{ef}	34 ± 0.38^{f}	54 ± 0.6^{c}	88 ± 0.9^{de}
200	235 ± 22^{ab}	$1{,}195\pm13.86^{c}$	$2{,}554\pm29^{c}$	$3{,}765\pm30.5^{d}$	3 ± 0.37^{def}	6 ± 0.8^{b}	10 ± 0.1^{e}	22 ± 0.25^{g}	24 ± 0.2^{e}	46 ± 0.4^{fgh}
300	214 ± 21^{ab}	726 ± 8.37^{e}	$3{,}223\pm37^{b}$	$3{,}959\pm3.75^{c}$	2 ± 0.21^{def}	2 ± 0.2^{bc}	4 ± 0.1^{fg}	$13\pm.0.15^{hi}$	68 ± 0.7^{b}	81 ± 0.8^{def}
500	224 ± 24^{ab}	275 ± 3.18^{h}	$6{,}913\pm79^{a}$	$7{,}192\pm138.2^{a}$	12 ± 3.69^{d}	27 ± 3.1^{a}	39 ± 0.4^{b}	36 ± 0.30^{f}	161 ± 1.8^{a}	197 ± 2.1^{c}
Methyl jasmonate (µM)										
100	235 ± 21^{ab}	695 ± 8.09^{f}	301 ± 3^{f}	$1{,}007\pm6.7^{g}$	16 ± 0.18^{c}	2 ± 0.02^{bc}	17 ± 0.2^{d}	367 ± 4.23^{a}	10 ± 0.1^{f}	385 ± 5.1^{a}
200	257 ± 47^{ab}	0	0	0	25 ± 0.28^{b}	0	25 ± 0.2^{c}	137 ± 1.58^{d}	0	139 ± 0.9^{cd}
300	264 ± 35^{ab}	$1{,}212\pm14.15^{c}$	0	$1{,}228\pm8.1^{f}$	8 ± 0.08^{de}	0	7 ± 0.1^{ef}	172 ± 1.99^{c}	0	174 ± 1.1^{bc}
500	212 ± 37^{ab}	91 ± 1.07^{j}	0	92 ± 0.6^{hi}	3 ± 0.03^{def}	0	2 ± 0.1^{fg}	10 ± 0.11^{h}	0	10 ± 0.1^{h}

Yeast extract (g/L)										
0.5	310 ± 36^{ab}	0	0	0	0	0	0	5 ± 0.06^{j}	0	6 ± 0.01^{h}
1	242 ± 25^{ab}	398 ± 4.63^{g}	602 ± 7^{c}	1005 ± 7.4^{g}	0	1 ± 0.01^{c}	1 ± 0.1^{fg}	6 ± 0.07^{ij}	16 ± 0.2^{f}	27 ± 5.3^{efgh}
2	272 ± 31^{ab}	821 ± 9.53^{d}	379 ± 4^{f}	1211 ± 7^{f}	1 ± 0.01^{f}	4 ± 0.05^{bc}	5 ± 0.1^{ef}	46 ± 0.52^{e}	34 ± 0.4^{d}	91 ± 11.5^{defg}
5	228 ± 28^{ab}	0	517 ± 6^{e}	517 ± 5.9^{j}	0	3 ± 0.02^{bc}	4 ± 0.1^{fg}	11 ± 0.13^{h}	28 ± 0.3^{de}	48 ± 9.4^{efgh}
Chitosan (mg/L)										
0	154 ± 15^{b}	182 ± 2.03^{i}	0	184 ± 1.2^{k}	1 ± 0.008^{f}	0	1 ± 0.1^{fg}	31 ± 0.35^{f}	0	31 ± 0.2^{efgh}
1	203 ± 40^{ab}	0	0	0	0	0	0	20 ± 0.22^{gh}	0	20 ± 0.1^{gh}
5	267 ± 55^{ab}	825 ± 9.53^{d}	0	835 ± 5.5^{h}	0	0	0	33 ± 0.38^{f}	0	33 ± 0.2^{efgh}
10	257 ± 53^{ab}	375±4.33g	0	380 ± 2.5^{j}	1 ± 0.01^{ef}	0	1 ± 0.1^{fg}	23 ± 0.26^{g}	0	23 ± 0.1^{fgh}

Values showed the mean ± standard error followed by similar letters in a column do not differ significantly at $p<0.05$

Control

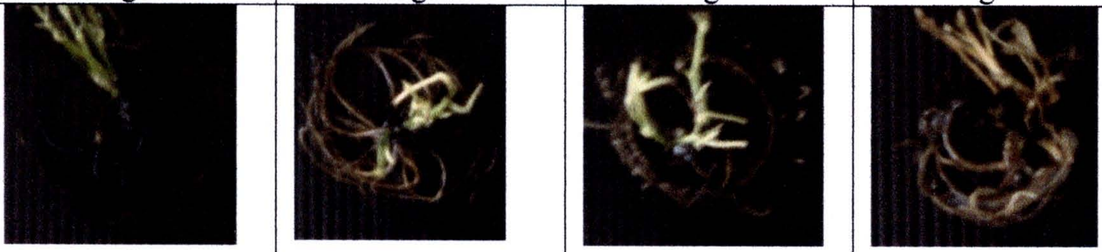


100mg/L SA

200mg/L SA

300 mg/L SA

500mg/L SA

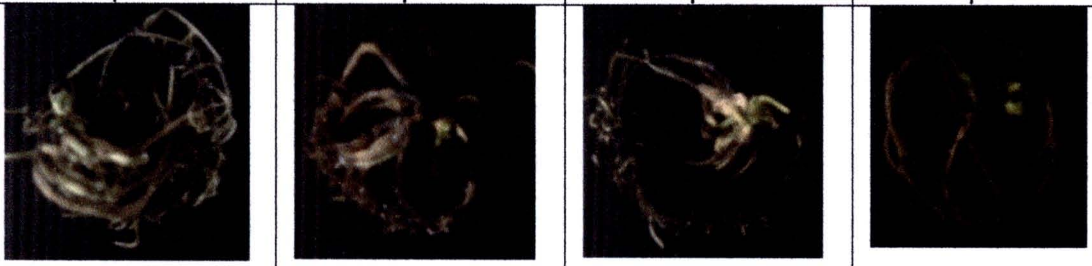


100 μM MJ

200 μM MJ

300 μM MJ

500 μM MJ



0.5 g/L Yeast

1 g/L Yeast

2 g/L Yeast

5 g/L Yeast

0 mg/L Chitosan

1 mg/L Chitosan

5 mg/L Chitosan

10 mg/L Chitosan

Figure 4.20 Effects of elicitors on cultured root

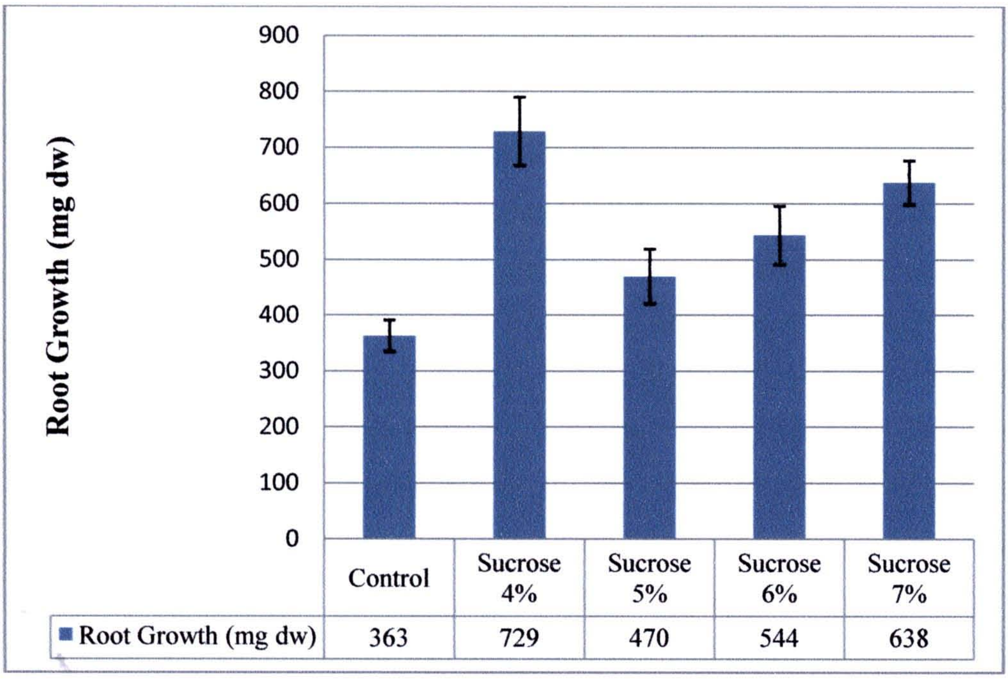
4.1.3 Effects of precursors on growth and *Stemona* alkaloids production in root cultures

Effects of sucrose on root growth

Sucrose was used with concentrations of 3, 4, 5 and 6%. The results showed the highest root growth production at 729±61 mg dw in the medium supplemented with 4% sucrose (Figure 4.21 and Table 4.3) with no effects on root browning (Figure 4.38). Osmotic agents such as sucrose not only acts a common source of carbon in the cell culture media (Al-Khayri and Al-Bahrany, 2002) and energy but also as an osmotica during organogenesis (Huang and Liu, 2002) and accumulated in many plant tissues in response to environmental stress, including water deficit (Ramos *et al.*, 1999) for playing a role in osmoregulation and cryoprotection. It has also been reported that sucrose in lower concentration (2% and 4%), is necessary for optimal growth and multiplication (George and Sherington 1984, Hazarika, 2003), decreases the fresh weight (Plas and Wagner, 1984), increases the dry weight (Kishore and Dange, 1990) but at high concentration reduces the growth rate (Shibli *et al.*, 1992). Osmotic potential is one of the most important parameter often affected by abiotic stresses. Under drought stress the osmotic potential in tolerant plants is reduced with the increasing intensity of stress. This reduced osmotic potential helps the plants to uptake more water and maintain growth (Almansouri *et al.*, 2000). Osmotic potential of the roots also increased with increasing stress level of the culture medium (Bajji *et al.*, 2000; Huang and Liu, 2002; Javed, 2002; Ahmad *et al.*, 2007), particularly by sucrose (Riham *et al.*, 2001). This increase in water potential and accumulation of osmotica like, total soluble carbohydrates were to accelerate the water uptake and hence enhance growth (Javed, 2002; Ahmad *et al.*, 2007).

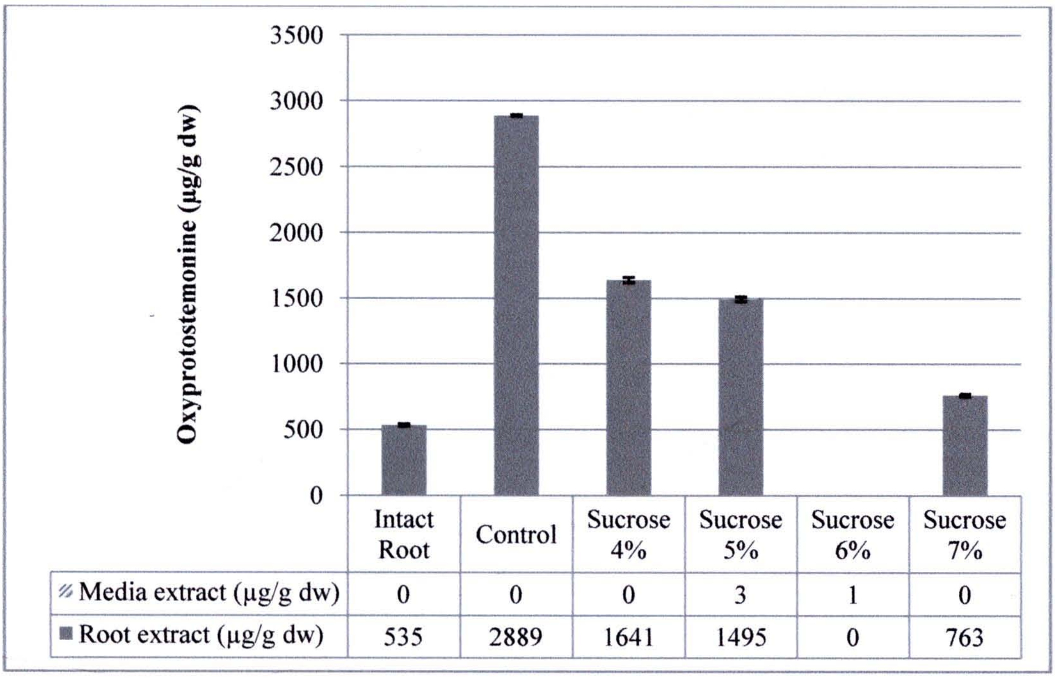
Effects of sucrose on oxyprotostemonine production

Increasing sucrose concentration resulted in decreased oxyprotostemonine accumulation. Figure 4.22 showed a maximum production of oxyprotostemonine in the 3% sucrose (control) at 2,889±33 μg/g dw, a 5 folds higher than that of intact root. Similar to Hilton and Rhodes (1990) reported that in hairy root cultures of *Datura stramonium*, in a bioreactor, 3% sucrose gave the best of root growth. Oxyprotostemonine alkaloid content decreased significantly with the increasing of sucrose concentration in the medium, surprisingly oxyprotostemonine was not detected at 6% sucrose level but increases again at 7% sucrose. There is not a clear reason to explain that fact, but it could be related to the water stress because additional sucrose to the medium can act as an osmotic agent that may introduce osmotic stress above certain concentrations. This can lead to a decrease in growth and alkaloid production (Kishore and Dange, 1990; Mehta *et al.,* 2000 and Kim and Kim, 2002). On another hand, oxyprotostemonine maybe converted to stemocurtisinol (Figure 4.24) or intermediate substrate so they were not detected at 6% sucrose.



	Control	Sucrose 4%	Sucrose 5%	Sucrose 6%	Sucrose 7%
■ Root Growth (mg dw)	363	729	470	544	638

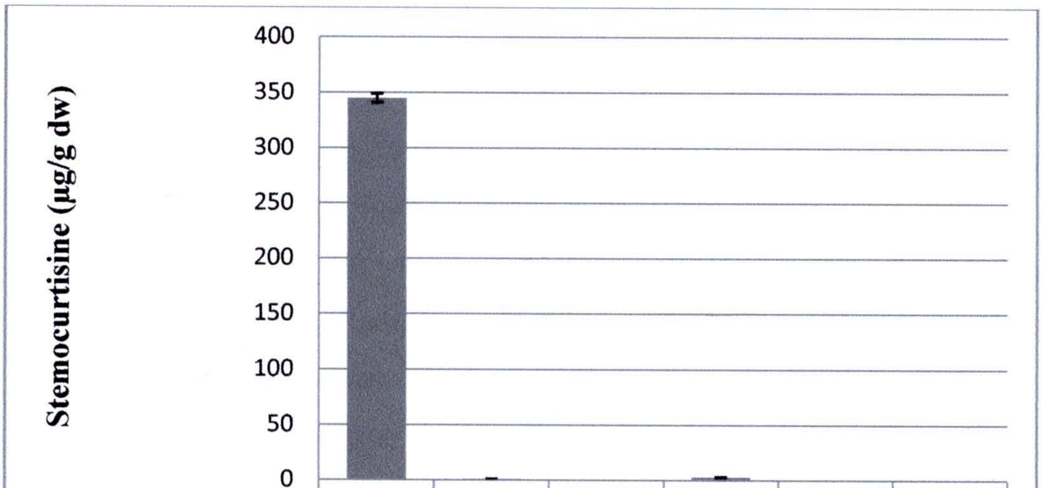
Figure 4.21 The effects of sucrose on root growth



	Intact Root	Control	Sucrose 4%	Sucrose 5%	Sucrose 6%	Sucrose 7%
▨ Media extract (µg/g dw)	0	0	0	3	1	0
■ Root extract (µg/g dw)	535	2889	1641	1495	0	763

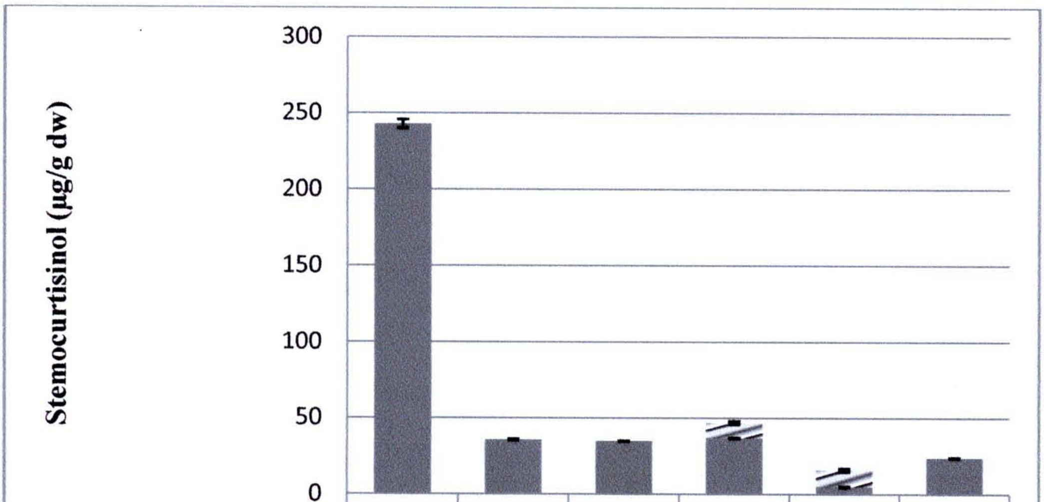
Figure 4.22 Effects of sucrose on oxyprotostemonine production.

The study on the effects of sucrose on stemocurtisine (Figure 4.23) and stemocurtisinol production (Figure 4.24) indicated that increasing the sucrose concentration had no effect on stemocurtisine and stemocurtisinol production. On the contrary, studies on *Hyoscyamus niger* cultures showed that increasing the sucrose concentration caused an increase in biomass as well as stimulation of scopolamine production (Hilton and Rhodes, 1994). Sucrose in the medium also increases photosynthesis in other cultured plants (Thic, 1992). Growth and alkaloid production in *Uncaria tomentosa* cell suspension cultures were studied. The increase of initial sucrose concentration up to 145 mM in the production medium enhanced the cell biomass by 3 folds and 58 mM sucrose concentration stimulated monoterpenoid oxindole alkaloids production in the maintenance medium (Luna-Palencia *et al.* 2005). Hairy roots cultures of *Hyoscyamus albus* were tested for growth and alkaloid production (5 tropane alkaloids simultaneously quantified by HPLC) in various media. Half-strength MS and Gamborg BS media containing 3% sucrose gave the highest alkaloid content (*ca* 2% dry wt), but only poor growth. On the other hand, fast growth was observed in woody plant medium containing 8% sucrose, while the production of tropane alkaloids was relatively low (Sauerwein and Shimomura, 1991).



	Intact Root	Control	Sucrose 4%	Sucrose 5%	Sucrose 6%	Sucrose 7%
▨ Media extract (µg/g dw)	0	0	0	0	0	0
■ Root extract (µg/g dw)	345	1	0	3	0	0

Figure 4.23 Effects of sucrose on stemocurtisine production.



	Intact Root	Control	Sucrose 4%	Sucrose 5%	Sucrose 6%	Sucrose 7%
▨ Media extract (µg/g dw)	0	0	0	10	11	0
■ Root extract (µg/g dw)	243	36	35	37	5	24

Figure 4.24 Effects of sucrose on stemocurtisinol production.

Effects of sodium acetate on root growth and alkaloid production

Sodium acetate was used at the concentrations of 20, 50 and 100 g/L. All treatments of sodium acetate had no effect on root growth (Figure 4.25). However, sodium acetate induced the browning of the roots (Figure 4.38). Treatments with sodium acetate, however, increased alkaloid production. Treatment of 20 mg/L sodium acetate enhanced production of oxyprotostemonine and stemocurtisinol to the highest extent at 5,052±58 μg/g dw (2 folds compared with control) (Figure 4.26) and 166±2 μg/g dw (5 folds higher than that of control) (Figure 4.28), respectively. Low concentrations of sodium acetate was used to enhance alkaloid production in other plants for example, the addition of 10 mg/L sodium acetate to the cell cultures of *Azadirachta indica* on day 3 also showed significantly increases in bioproduction of azadirachtin (Balaji, 2003). While a relatively high concentration (50 mg/L) of sodium acetate was optimal for stemocurtisine production (21±0.01 μg/g dw, 21 folds compared with control) (Figure 4.27). Similarly, the addition of 50 mg/L of sodium acetate to callus cultures of *Artemisia annua*, on the 10th day resulted in the production of 32.2 mg/L artemisinin (Baldi and Dixit, 2008)

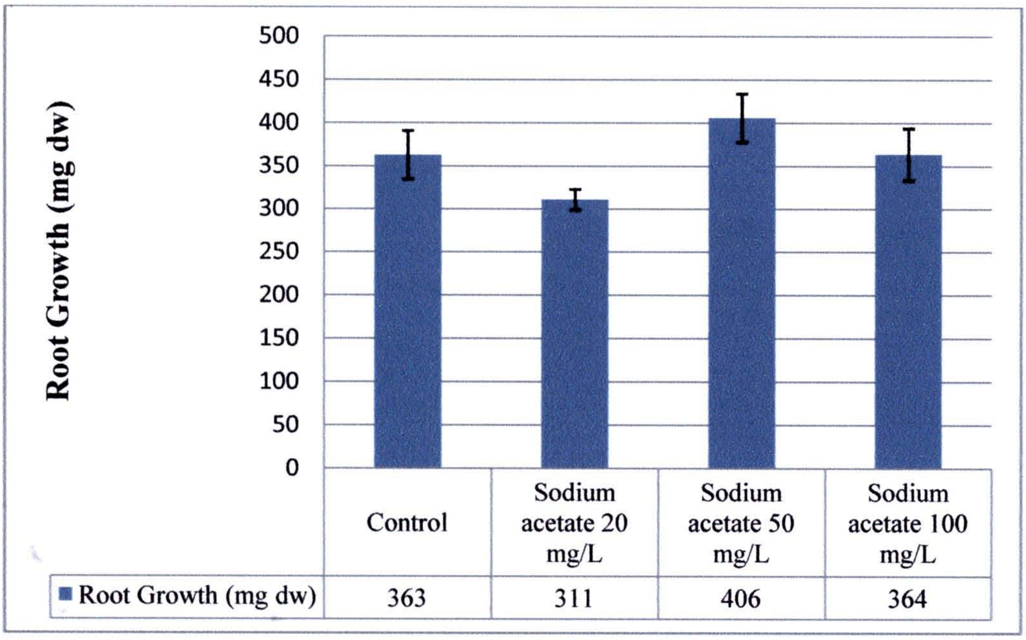
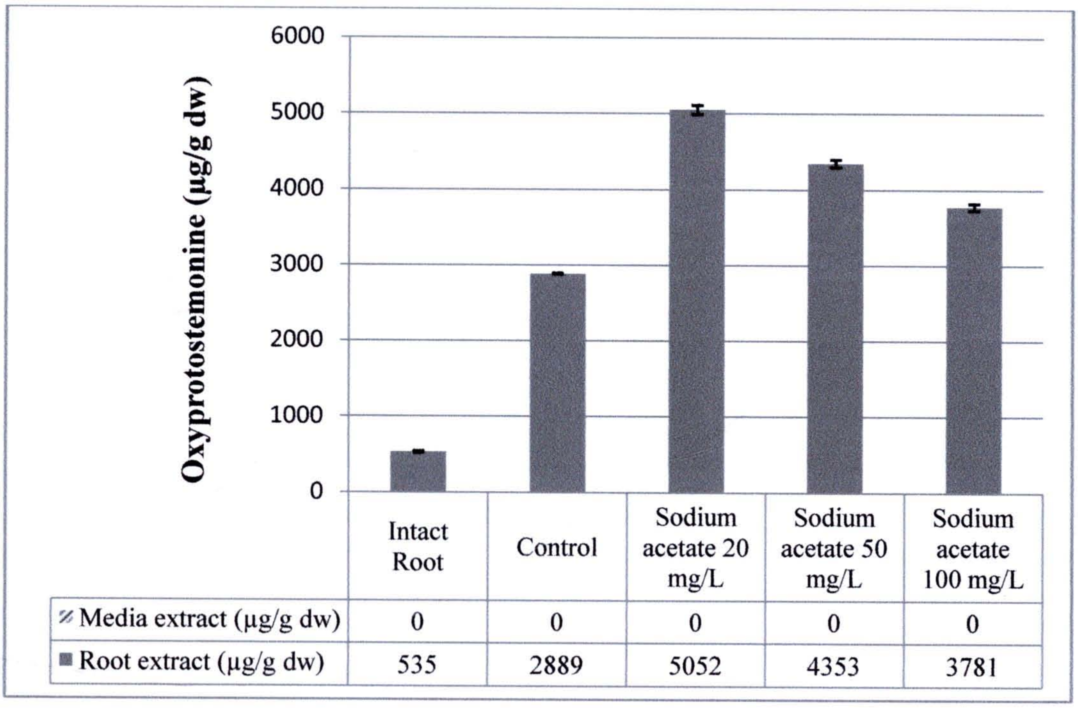
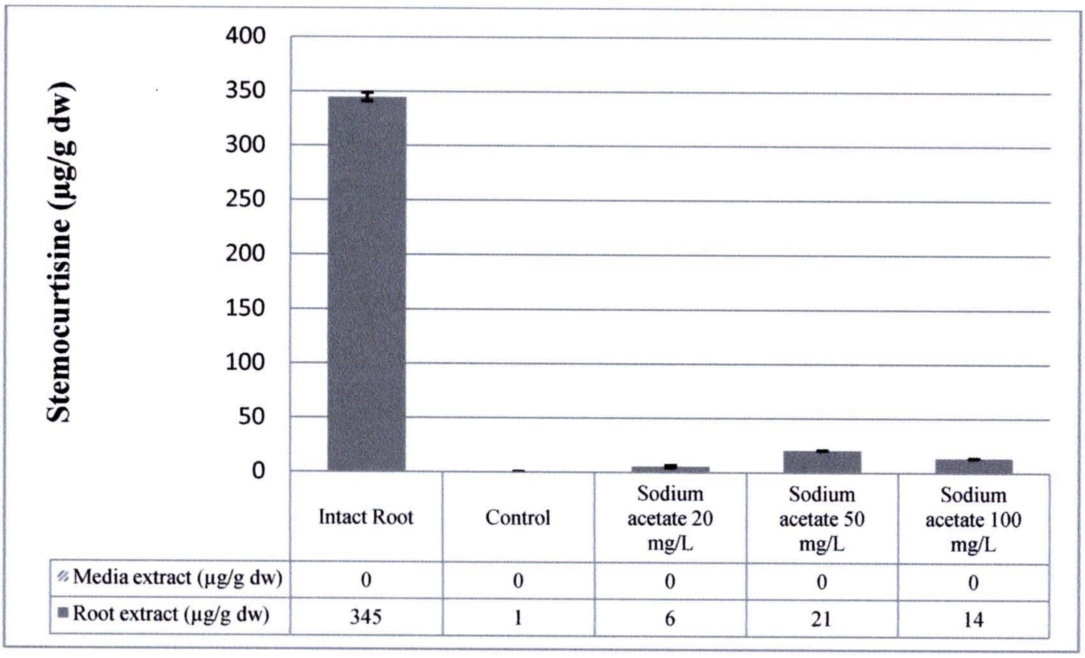


Figure 4.25 Effects of sodium acetate on root growth



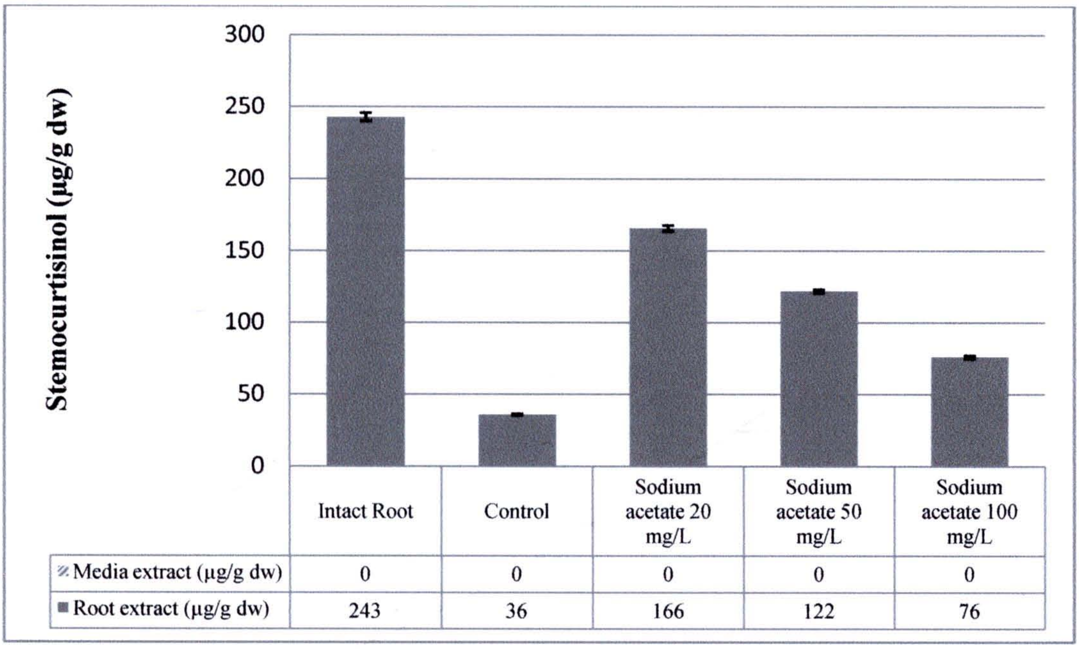
	Intact Root	Control	Sodium acetate 20 mg/L	Sodium acetate 50 mg/L	Sodium acetate 100 mg/L
▨ Media extract (µg/g dw)	0	0	0	0	0
■ Root extract (µg/g dw)	535	2889	5052	4353	3781

Figure 4.26 Effects of sodium acetate on oxyprotostemonine production.



	Intact Root	Control	Sodium acetate 20 mg/L	Sodium acetate 50 mg/L	Sodium acetate 100 mg/L
▨ Media extract (µg/g dw)	0	0	0	0	0
■ Root extract (µg/g dw)	345	1	6	21	14

Figure 4.27 Effects of sodium acetate on stemocurtisine production.

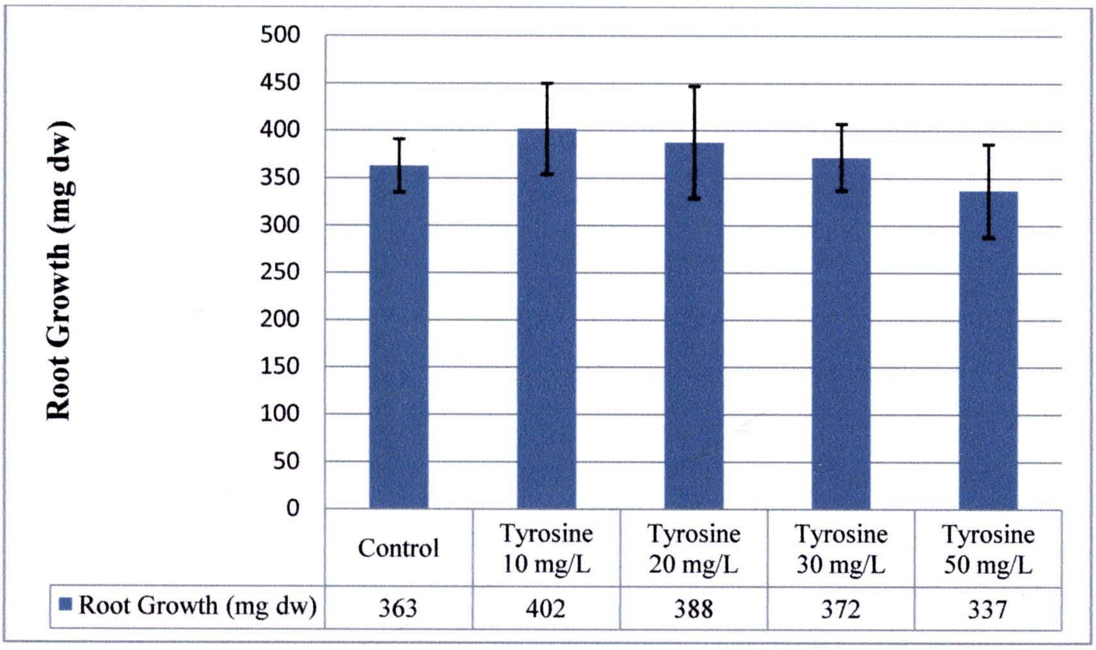


	Intact Root	Control	Sodium acetate 20 mg/L	Sodium acetate 50 mg/L	Sodium acetate 100 mg/L
▨ Media extract (µg/g dw)	0	0	0	0	0
■ Root extract (µg/g dw)	243	36	166	122	76

Figure 4.28 Effects of sodium acetate on stemocurtisinol production.

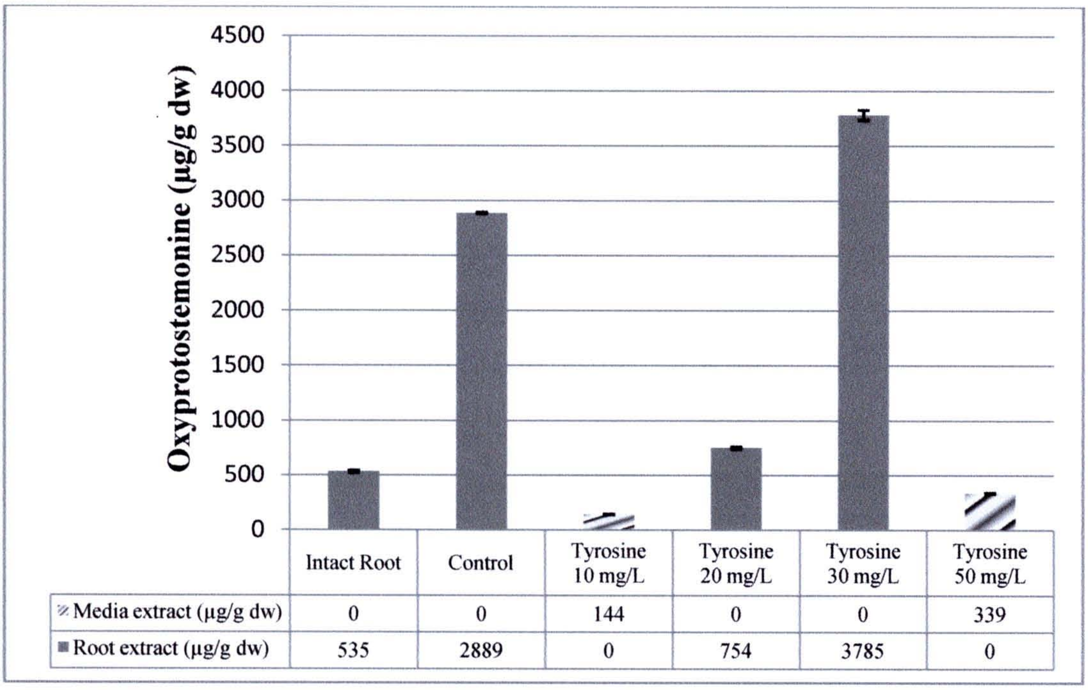
Effects of tyrosine on root growth and alkaloid production

Tyrosine is an amino acid precursor for the production of aporphine alkaloids in the biosynthesis pathway (Putalum *et al.*, 2009). Therefore, we tested the effects of tyrosine on the growth of roots and alkaloid content. The biomass of roots slightly decreased (not significantly) for all tyrosine concentrations tested (Figure 4.29). Figure 4.30 shows that the highest oxyprotostemonine content (3,785±45 µg/g dw, 7 folds higher than that of intact root) was found upon treatment of 30 mg/L tyrosine. On the other hand, the maximum stemocurtisine and stemocurtisinol accumulation was observed using 20 mg/L tyrosine (Figures 4.31 and 4.32). This finding suggested that the utilization of tyrosine can enhance alkaloid production. These results were similar to those found in previous studies on hairy roots cultures of *Rhodiola sachalinensis* (Zhou *et al.*, 2007). On the basis of these results tyrosine may be incorporated into the alkaloid pathway, acting directly as a precursor of tyramine synthesis, since tyrosine has been considered to be a precursor for numerous secondary metabolites (Oliveira and Machado, 2003).



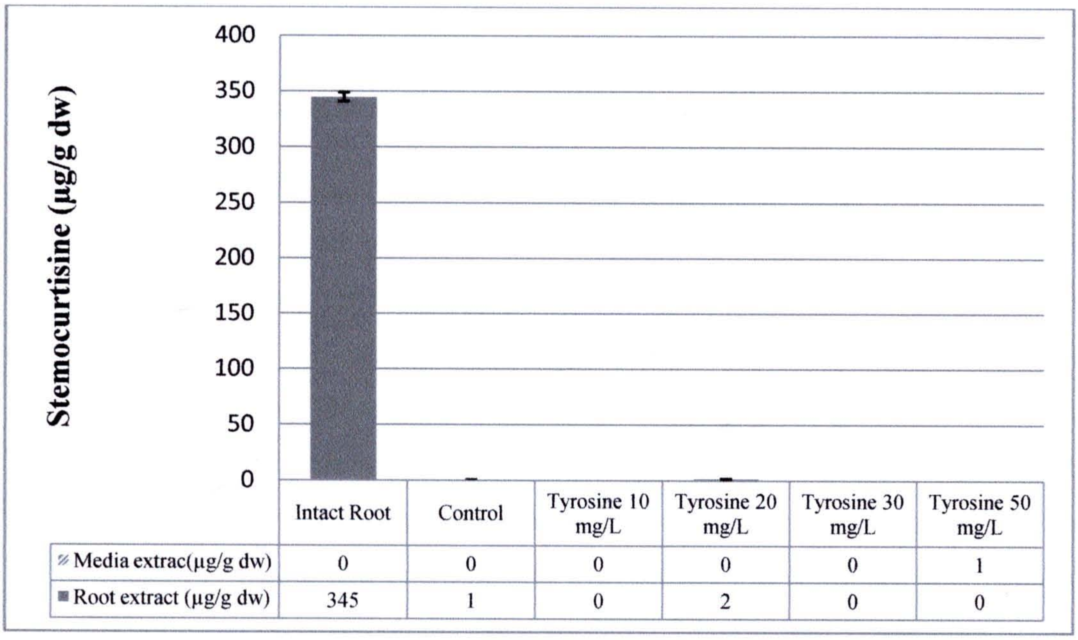
	Control	Tyrosine 10 mg/L	Tyrosine 20 mg/L	Tyrosine 30 mg/L	Tyrosine 50 mg/L
■ Root Growth (mg dw)	363	402	388	372	337

Figure 4.29 Effects of tyrosine on root growth



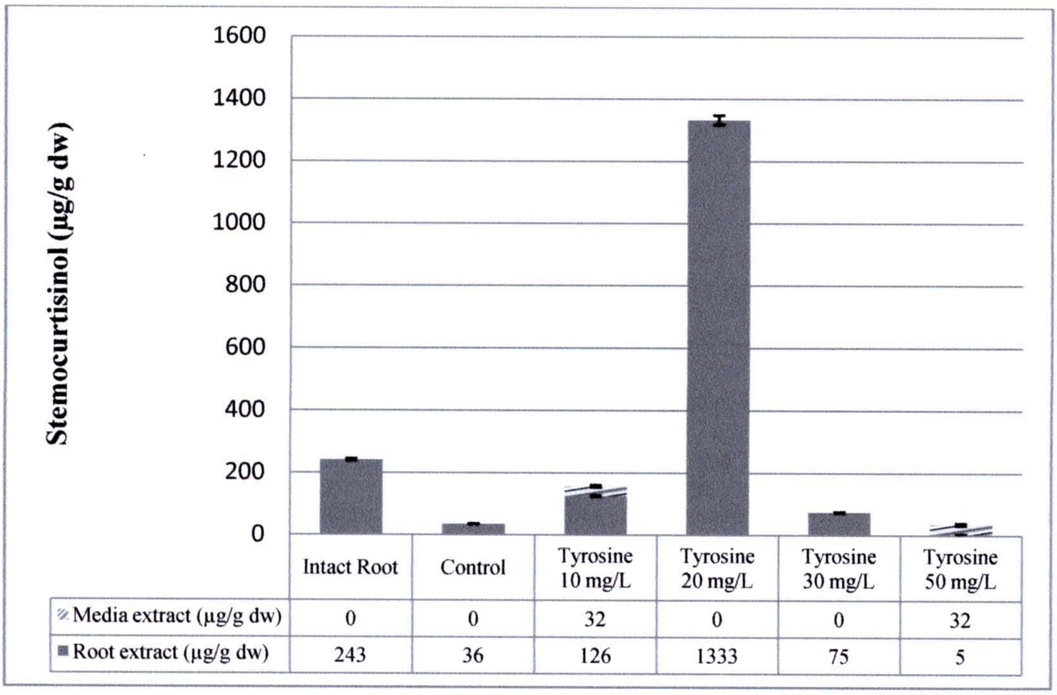
	Intact Root	Control	Tyrosine 10 mg/L	Tyrosine 20 mg/L	Tyrosine 30 mg/L	Tyrosine 50 mg/L
▨ Media extract (µg/g dw)	0	0	144	0	0	339
■ Root extract (µg/g dw)	535	2889	0	754	3785	0

Figure 4.30 Effects of tyrosine on oxyprotostemonine production.



	Intact Root	Control	Tyrosine 10 mg/L	Tyrosine 20 mg/L	Tyrosine 30 mg/L	Tyrosine 50 mg/L
▨ Media extrac(µg/g dw)	0	0	0	0	0	1
■ Root extract (µg/g dw)	345	1	0	2	0	0

Figure 4.31 Effects of tyrosine on stemocurtisine production.



	Intact Root	Control	Tyrosine 10 mg/L	Tyrosine 20 mg/L	Tyrosine 30 mg/L	Tyrosine 50 mg/L
▨ Media extract (µg/g dw)	0	0	32	0	0	32
■ Root extract (µg/g dw)	243	36	126	1333	75	5

Figure 4.32 Effects of tyrosine on stemocurtisinol production.

4.1.4. Effects of culture condition on root growth and alkaloid production

Effects of the temperature on root growth and alkaloid production

To investigate the effects of temperature on root growth and alkaloid production, the roots were cultured at 20, 25 (control) and 30 °C. Figures 4.33 show that temperature had no effects on root growth and alkaloid production (Figures 4.34, 4.35 and 4.36). However, the 30 °C cultures resulted in the formation of hairy roots (Figure 4.39). In contrast, Iranbakhsh (2007) reported the effects of different temperatures on cell growth and hyocyamin production in *Datura stramonium* cell cultures. The results showed the maximum content of alkaloid production was at 20 °C with the highest fresh and dry weight of the biomass observed at 25 °C. Similarly, Rhoton and Bouteraouy (1994) reported that hyocyamin production in tissue cultures of *Datura stramonium* at 20 or 25 °C increases was higher than that at 30 °C.

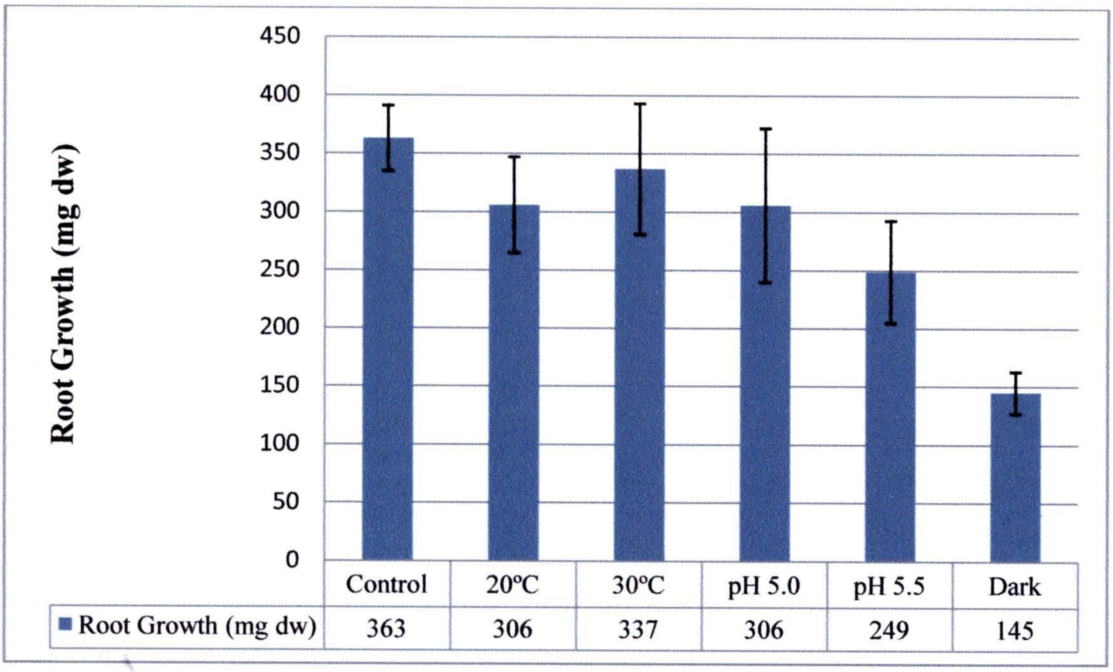
Effects of the initial pH on the medium on root growth and alkaloid production

To investigate the effects of the pH of the medium on root growth and alkaloid production in *S. curtisii* root culture, the roots were grown in media at different pH values, ranging from 5.0-5.8. Figure 4.33 shows that the pH of the medium did not have a signification effect on the dry weight of the root (Table 4.3), but apparently affected the oxyprotostemonine content (Figure 4.34). The maximum total oxyprotostemonine content was 4,142±48 μg/g dw (43% higher than control and 8 folds higher than that of intact root) at pH 5.0. These results were similar to those involving decreasing the pH of the medium, which slightly increased (not significantly) stemocurtisinol production (Figure 4.36). Although previous research has reported that the pH had an effect on growth and secondary metabolite production in the roots of different plant species (Mukundan and Hjorsto 1991). However, decreasing the medium pH to 5.0 and 5.5 had no effect on production of stemocurtisine (Figure 4.35).

Effects of the illumination on root growth and alkaloid production

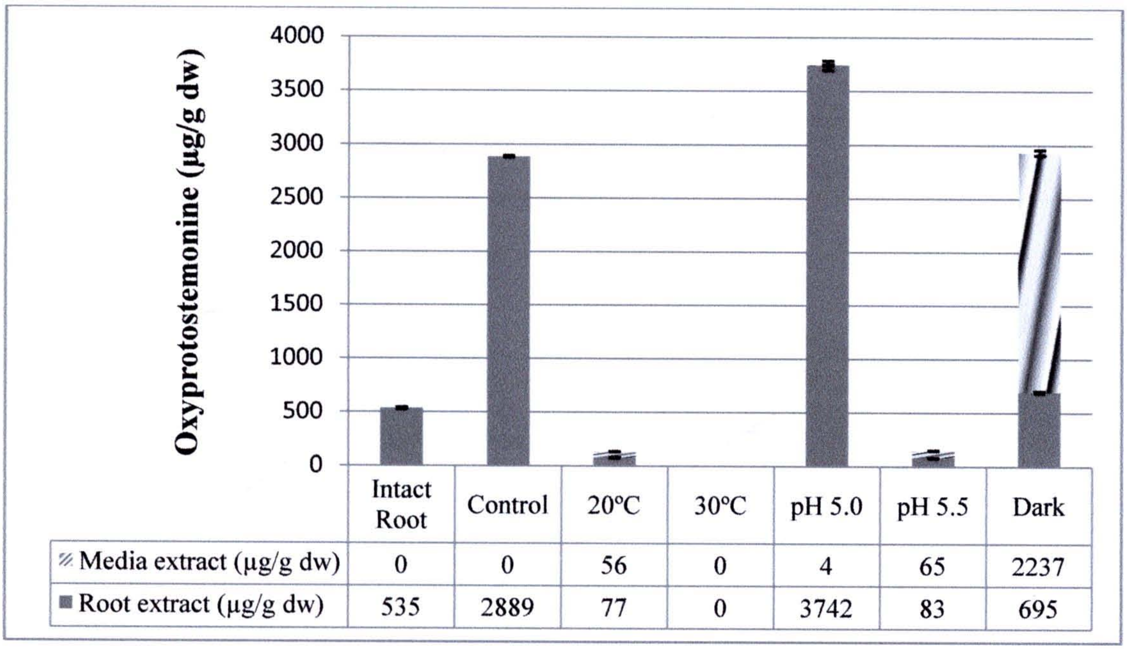
Inhibition of root growth observed when incubated under dark

The root weight obtained from cultures grown under the dark were relatively less (145±18 mg dw) than those of the roots grown under illumination (control), with significant differences (Figure 4.33). Induction of morphogenesis in the roots was found to be associated with irradiance. Prominent greenish shoot buds appeared on the roots (Figure 4.39). Roots grown under dark conditions had no effect on alkaloid production but induced alkaloid secretion into the medium (Figures 4.34, 4.35 and 4.36). In contrast to light-induced root growth inhibition (Mukundan and Hjortsa, 1991) there was an increase in secondary metabolite production as compared to dark-grown hairy root cultures in other plant (Hagimori *et al.* 1982 and Ohlsson *et al.* 1983).



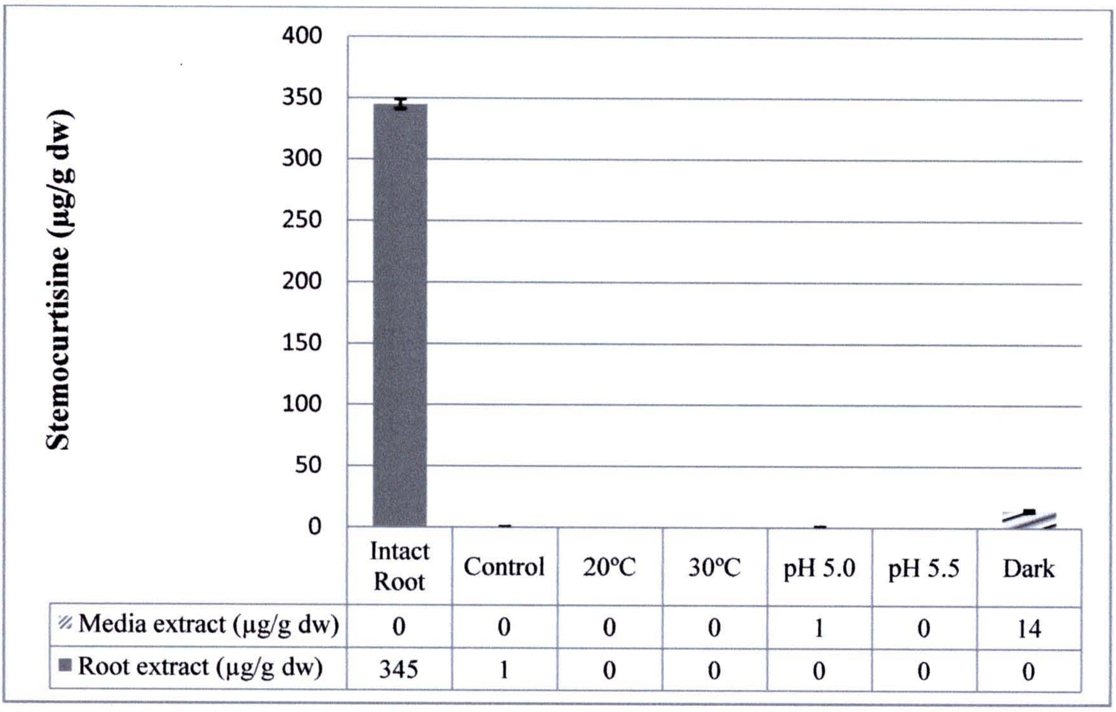
	Control	20°C	30°C	pH 5.0	pH 5.5	Dark
■ Root Growth (mg dw)	363	306	337	306	249	145

Figure 4.33 Effects of culture conditions on root growth



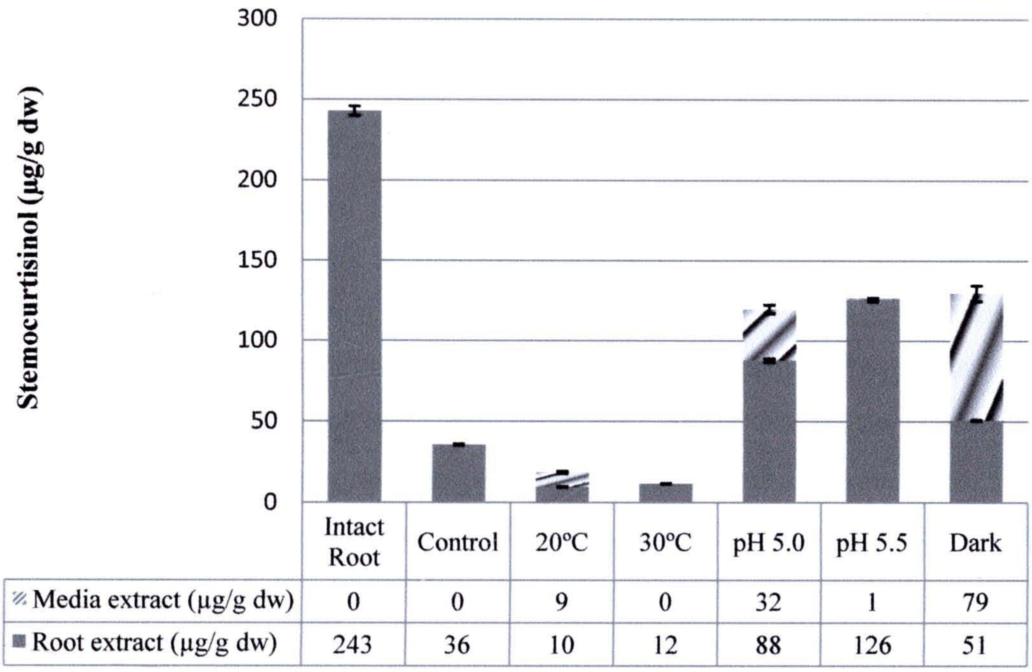
	Intact Root	Control	20°C	30°C	pH 5.0	pH 5.5	Dark
▨ Media extract (µg/g dw)	0	0	56	0	4	65	2237
■ Root extract (µg/g dw)	535	2889	77	0	3742	83	695

Figure 4.34 Effects of culture conditions on oxyprotostemonine production.



	Intact Root	Control	20°C	30°C	pH 5.0	pH 5.5	Dark
▨ Media extract (µg/g dw)	0	0	0	0	1	0	14
■ Root extract (µg/g dw)	345	1	0	0	0	0	0

Figure 4.35 Effects of culture conditions on stemocurtisine production.



	Intact Root	Control	20ºC	30ºC	pH 5.0	pH 5.5	Dark
▨ Media extract (μg/g dw)	0	0	9	0	32	1	79
■ Root extract (μg/g dw)	243	36	10	12	88	126	51

Figure 4.36 Effects of culture conditions on stemocurtisinol production.

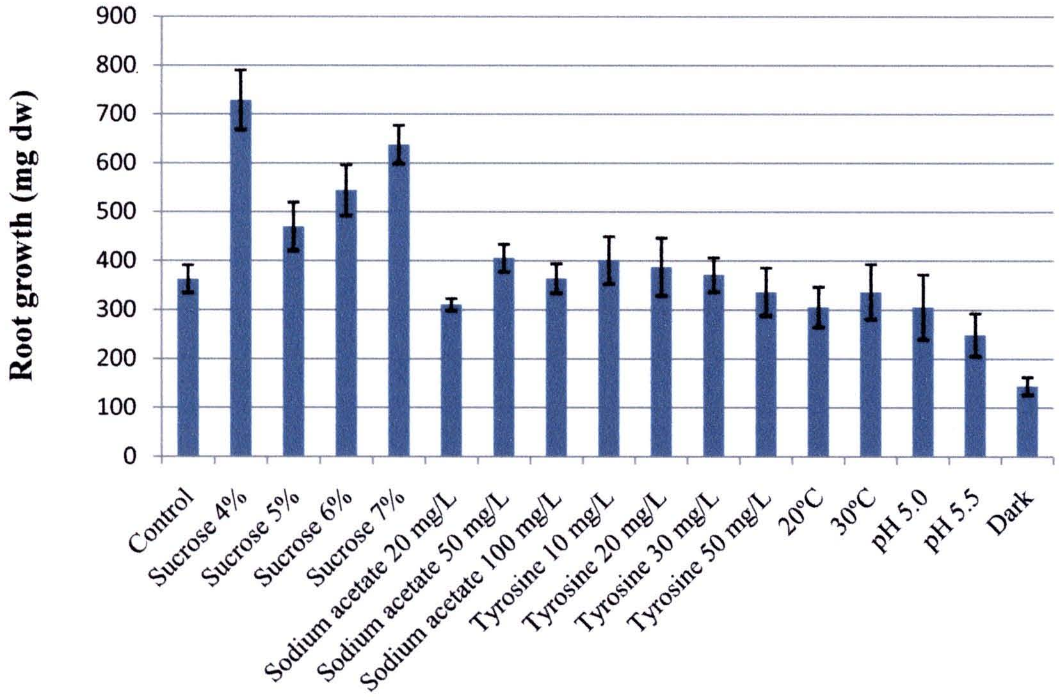


Figure 4.37 Effects of precursors and culture conditions on root growth.

Conclusion

Figure 4.37 and Table 4.3 demonstrate the effects of precursors and culture conditions on root growth after cultured for 16 weeks. The results investigated that all of precursor treatments had no effects on root growth. Exception the treatment of 4% and 7% sucrose show significantly increased root growth when compared with control. Treatment of culture conditions show non-significantly difference root growth with control. Only root grown in dark condition had significantly lower root growth than that of control. The morphology of roots grown in precursors were showed in Figure 4.38. Roots were also light-green-colored and formed tuberous roots whereas roots treated with different culture conditions, light-green-colored roots turned brown and occurred in media pH 5.0 and 5.5 (Figure 4.39). Furthermore, roots cultured at 30°C stimulated hairy root proliferation (Figure 4.39). The effects of precursors and culture conditions on *Stemona* alkaloids production were showed in Table 4.3. The highest of oxyprotostemonine accumulation in cultured roots were detected at 1333±15 μg/g dw from roots treated with 20 mg/L tyrosine.

Table 4.3 Influence of precursors and culture conditions on the alkaloid content in root and cultures media of *S. curtisii*

Influence of precursors and culture conditions on the alkaloid content in root and cultures media of *S. curtisii*.

		Alkaloid content								
	Root Growth	**Oxyprotostemonine (µg/g dw)**			**Stemocurtisine (µg/g dw)**			**Stemocurtisinol (µg/g dw)**		
	(mg dw)	**Root extract**	**Media extract**	**Total**	**Root extract**	**Media extract**	**Total**	**Root extract**	**Media extract**	**Total**
Intact root	-	535±9g	-	535±6h	345±4a	-	345±17.2a	243±3b	-	243±3b
Control	363±28cde	2,889±6d	0	2,889±33e	1±0.1de	0	1±0.1de	36±0.01fg	0	36±0.01fg
Sucrose (%)										
4	729±61a	1,641±19e	0	1,641±19f	0	0	0	35±0.01fg	0	35±0.01fg
5	470±49bcd	1495±18e	3±0.2d	1,673±19f	3±0.1de	0	3±0.1de	37±0.01fg	10±0.9a	47±0.9efg
6	544±52abc	0	1±0.01fg	32±0.1j	0	0	0	5±0.01i	11±1a	16±0.11g
7	638±39ab	763±9f	0	763±9g	0	0	0	24±0.02gh	0	24±0.02g
Sodium acetate (mg/L)										
20	311±12de	5,052±58a	0	5,052±58a	6±0.1d	0	6±0.3d	166±2c	0	166±2bc
50	406±28cd	4,353±50b	0	4,353±50b	21±0.01b	0	21±10b	122±1d	0	122±1cdef
100	364±30cde	3,781±44c	0	3,781±44d	14±0.01c	0	14±0.7c	76±1e	0	76±1cdefg

Tyrosine (mg/L)										
10	402±48cd	0	144±2e	144±2j	0	0	0	126±1d	32±2.7a	158±28bcd
20	388±59cd	754±9f	0	754±9g	2±0.1de	0	2±0.1de	1333±15a	0	1333±15a
30	372±35cd	3,785±45c	0	3,785±45d	0	0	0	75±1e	0	75±1defg
50	337±49cde	0	339±4c	339±4i	0	1±0.1bc	1±0.1de	5±0.01i	32±2.6a	37±26fg
Temperature (°C)										
20	306±41de	77±1h	56±1f	133±1j	0	0	1±0.1e	10±0.1d	9±0.7a	19±7g
30	337±56cde	0	0	0	0	0	0	12±0.1i	0	12±0.1g
Media pH										
5.0	306±66de	3,742±43c	400±5b	4,142±48c	0	1±0.1b	1±0.1de	88±1e	32±2.6a	120±27cdef
5.5	249±44de	83±1h	65±1f	148±2j	0	0	0	126±1d	1±0.1a	127±2cde
Dark (24hr/d)	145±18e	695±8f	2,237±26a	2,933±34c	0	14±1a	14±0.7c	51±0.1f	79±4.9a	130±5cde

Values showed the mean ± standard error followed by similar letters in a column do not differ significantly at $p<0.05$

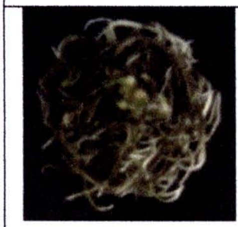
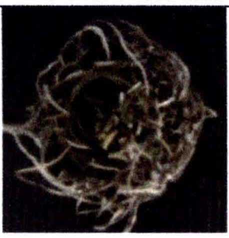
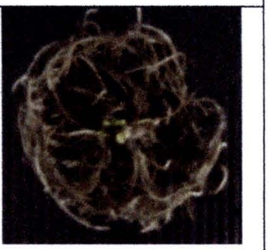
			
Control			
			
4% sucrose	5% sucrose	6% sucrose	7% sucrose
			
20 mg/L Sodium acetate	50 mg/L Sodium acetate	100 mg/L Sodium acetate	
			
10 mg/L tyrosine	20 mg/L tyrosine	30 mg/L tyrosine	50 mg/L tyrosine

Figure 4.38 Effects of precursors on cultured roots.

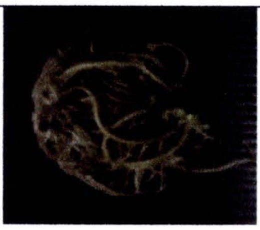
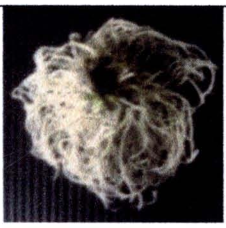
	
Control	Dark
	
20 °C	30 °C
	
pH 5.0	pH 5.5

Figure 4.39 Effects of culture conditions on cultured roots.

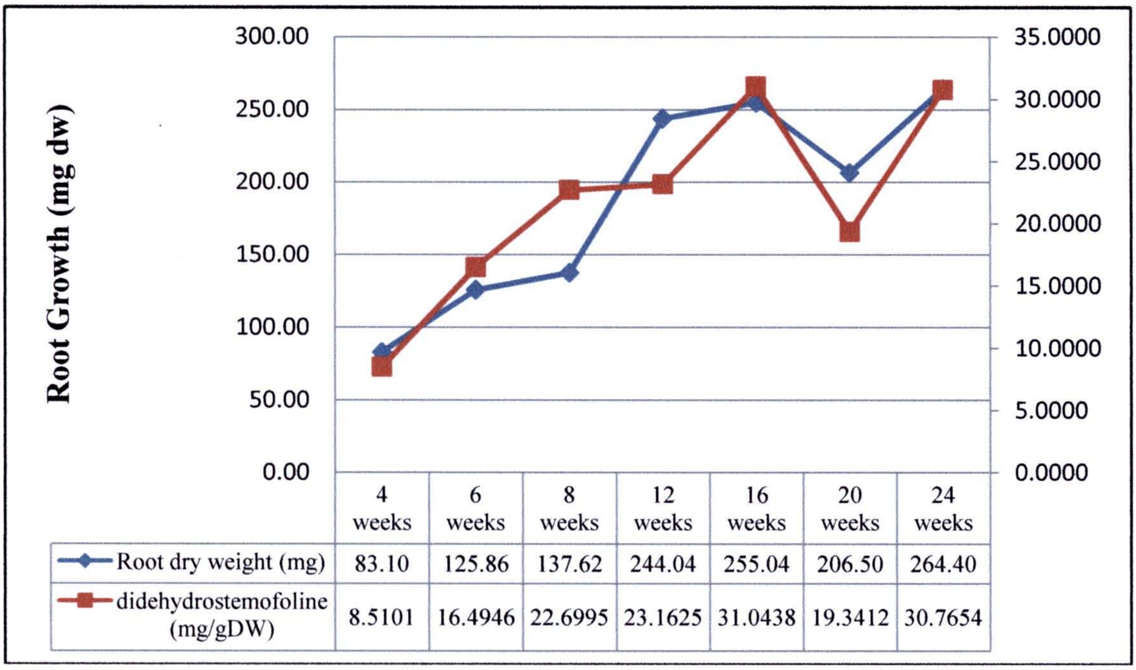
4.2 Time profile study of 1′,2′-didehydrostemofoline production in root cultures of *Stemona* sp.

Figure 4.32 and Table 4.4 illustrates the time course growth and 1′,2′-didehydrostemofoline accumulation in root cultures of *Stemona* sp. The lag phase was about 8 weeks and the linear growth phase was between weeks 8 -12, followed by a stationary phase between weeks 12-16. After the stationary phase, in which nutrients have been depleted, root dry weight showed a steady decline. The maximum root dry weight (255 mg dw) was obtained at the end of the stationary phase at week 16, which also corresponded to the highest 1′,2′-didehydrostemofoline production (31.04 mg/g dw) in the root cultures. However, the 1′,2′-didehydrostemofoline production at this time was not different statistically from the production of 24 weeks old root. It seems that 1′,2′-didehydrostemofoline accumulation occurred parallel to the root growth pattern. The pattern of root growth and alkaloid production was similar to *Salvia miltiorrhiza* hairy roots culture. The increase of total tanshinone content and the root dry weight went together (Wu and Shi, 2008). The results investigated that 1′,2′-didehydrostemofoline was produced by *in vitro* root cultures of *Stemona* sp. Similar to Sastraruji (2010) reported the isolation of 1′,2′-didehydrostemofoline, as the major alkaloid from roots extracts of this *Stemona* species growing naturally in Amphur Mae Moh, Lampang and in Amphur Chattrakarn, Phitsanulok Thailand (Sastraruji *et al.*, 2005). However, it was found that natural root could produce this alkaloid at 47.46 mg/g dw (53% higher than that of cultured root at 16 weeks). Therefore, this plant will not be studied the effects of precursors, elicitors and culture condition.

Table 4.4 Time profile of 1′,2′-didehydrostemofoline production.

Time periods (weeks)	1′,2′-didehydrostemofoline (mg/g dw)
4	8.51±0.30^{e}
6	16.49±0.40^{d}
8	22.70±1.13^{b}
12	23.16±0.40^{b}
16	31.04±1.62^{a}
20	19.34±0.27^{c}
24	30.76±0.17^{a}

1′,2′-didehydrostemofoline content is represented by mean ± standard deviation, $p<0.05$.



	4 weeks	6 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
◆ Root dry weight (mg)	83.10	125.86	137.62	244.04	255.04	206.50	264.40
■ didehydrostemofoline (mg/gDW)	8.5101	16.4946	22.6995	23.1625	31.0438	19.3412	30.7654

Figure 4.40 Time profile of root growth and 1′,2′-didehydrostemofoline production from *Stemona* sp. root cultures