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Changes in Solasodine Accumulation in Regenerated Plants of *Solanum nigrum* Transformed with *Agrobacterium rhizogenes* 15834

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Abstract: The objective of this research was to determine the profile, distribution and solasodine contents in the transformed plants compared to the control plants. Transgenic plants were regenerated spontaneously *in vitro* from *Agrobacterium rhizogenes*-transformed root cultures of *Solanum nigrum* L. (black nightshade). Upon transfer to greenhouse, all the transformed root-derived plants were fertile with seed set considerably reduced compared to the control plants. The seeds harvested from the mature plants were used to study the steroidal alkaloid solasodine productivity during 16 week of plant development in greenhouse. Chromatographic analyses (TLC and HPLC) and IR spectrophotometry analysis showed that the solasodine profile in the transformed plants was similar to the solasodine profile in control plants. Solasodine contents in plant parts of the transformed plants were lower compared to that of the control plants. The solasodine contents in the control plants were 9.93 mg g^{-1} (roots), 6.10 mg g^{-1} (stems), 4.06 mg g^{-1} (leaves) and 0.61 mg g^{-1} (fruits), whereas the solasodine contents in the transformed plants were 7.75 mg g^{-1} (roots), 2.12 mg g^{-1} (stems), 3.98 mg g^{-1} (leaves) and 0.71 mg g^{-1} (fruits). Solasodine was differently distributed in the transformed plants compared to the control plants. In control plants, the highest solasodine accumulation was occur in stems (78.1%), whereas in transformed plants the highest solasodine accumulation was occur in leaves (51.6%). It seems that the presence of *rol* (A, B, C and D) genes contained in the T_L T-DNA is detrimental to the solasodine accumulation in the transformed plants in contrast to transformed root cultures.

Key words: *Agrobacterium rhizogenes*, solasodine, *Solanum nigrum* L., transformed plant

INTRODUCTION

Hairy roots have been investigated as a biological system for the production of valuable compounds from medicinal plants for over 25 years. However, in general the hairy root technology is not economically feasible due to high production cost and scaling-up problems. There is only few examples on the commercial phytochemical production using hairy roots, e.g., production of camptothecin and podophyllotoxin by German company ROOTec (Guillon *et al*., 2006). An alternative production method with relatively low cost is by using hairy root-derived plants. This method may be more suitable to be applied in developing countries such as Indonesia where land availability is not a problem.

Solanum plants and their cell and tissue cultures contain valuable steroidal alkaloids such as solasodine (Jacob and Malpathak, 2005; Lee *et al*., 2007). Hairy root cultures of *Solanum* spp. represent differentiated, genetically transformed organ cultures that produce high levels of the same alkaloids as are found in the intact plants (Subroto and Doran, 1994; Subroto *et al*., 1996; Jacob and Malpathak, 2004, 2005). Recent work shows that plant regeneration can also enhance solasodine productivity in hairy root cultures of *Solanum khasianum* (Jacob and Malpathak, 2005). Therefore, we were interested in whether plants could be regenerated from hairy roots and whether the productivity of secondary metabolite synthesis is enhanced, maintained or reduced in regenerated plants compared to hairy root cultures or wild-type plants.

From previous research, transgenic plants were regenerated spontaneously *in vitro* from *Agrobacterium rhizogenes*-transformed root cultures of *Solanum nigrum* L. (black nightshade). Upon transfer to greenhouse, all the transformed root-derived plants were fertile with seed set considerably reduced compared to the control plants. PCR analyses showed that only T_L T-DNA was integrated and expressed into the plant genome (Subroto *et al*., 2001). This expression was remain stable over 6 years of storage and the seeds were also remain viable during the same period. The seeds harvested from the mature plants were

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then used to study the steroidal alkaloid solasodine productivity during 16 week of plant development in greenhouse. Thus, the objective of this research was to determine the profile, distribution and solasodine contents in the transformed plants compared to the control plants.

MATERIALS AND METHODS

General: HPLC instrument used was JASCO PU-980 intelligent HPLC Pump with JASCO UV-970 intelligent UV/Vis detector including JASCO 807-IT integrator. The column was Bondapak C_{18} (300 ×3.9 mm). The Infra Red spectrophotometer (IR) used was Bio Rad FTS 3000 Excalibur Series. Authentic solasodine was purchased from Sigma.

Plant materials: Normal (wild-type) and transgenic seeds of *S. nigrum* L. used in this experiment were harvested from the regenerated plants as described previously (Subroto *et al*., 2001). Plant regeneration experiment was conducted in a greenhouse in Cibinong, Indonesia, in the early 2004 for 16 weeks. The seeds were regenerated in pots in greenhouse with medium composition of soil:compost = 1: 1. After 16 week of plant development, the regenerated plants were harvested. Roots, stems, leaves and fruits were separated from each plant for determination of dry weights and solasodine contents. The experiment was conducted in triplicate. Plant determination was conducted by Herbarium Bogoriense, Bogor and the results showed that both normal and transgenic plants registered to Herbarium Bogoriense were *Solanum nigrum* L.

Extraction: All samples were dried under sunlight and grounded to powders. Ca 100 mg of each sample was extracted according to the method as described previously (Sharp and Doran, 1990).

TLC analyses: One milliliter of each extract was extracted with 5 mL of ethanol-free chloroform and the lower phase (chloroform phase) was separated. Authentic solasodine was dissolved in ethanol-free chloroform, then the samples and authentic solasodine solutions were spotted on TLC plates (silica gel GF_{254}) and eluted with mobile phase n-hexane:methanol:acetone = 8: 1: 1. The plates were then dried and sprayed with cerium sulphate in sulphuric acid, then heated at 110°C for ca. 10 min.

HPLC analyses: One milliliter of each extract was added to 5 mL ethanol-free chloroform, mixed and the lower phase was removed and evaporated to dryness and dissolved in the mobile phase methanol: Tris buffer 0.01 M = 75:25 up to 5.0 mL. Twenty micro liter of the sample solution was injected into HPLC with conditions as follows: Ambient temperature, flowrate: 1.00 mL min^{-1}, mobile phase: Methanol: Tris buffer 0.01 M (pH 7.0) = 75:25, detector: UV/Vis, wavelength: 205 nm.

FTIR analysis: Ca. 1-2 mg of sample was added with 300 mg potassium bromide and grounded, then injected to the Fourier Transform Infra Red (FTIR) instrument. IR spectra was used to determine the functional groups in solasodine presence in the samples.

RESULTS AND DISCUSSION

TLC analyses were used to detect the presence of solasodine in every part of the plants qualitatively. In addition, this method was used to analyze the solasodine profiles in the normal and transgenic plants. The observation was conducted qualitatively by comparing the colour of the sample spots with authenitc solasodine as well as their Rf values. The results showed that the chloroform extract of each sample contained solasodine with Rf values ca. 0.150-0.175. The solasodine profile of normal plants was also similar to the solasodine profile of transgenic plants.

The results show that the highest solasodine contents in normal as well as transgenic plants were found in roots, i.e., 9.93 and 7.75 mg g^{-1}, respectively (Table 1 and 2). This was due to the facts that most of solasodine is synthesized and stored in roots and then distributed to other plant parts such as stems, leaves and fruits. However, from these results it is still cannot be concluded that roots are the main source of solasodine synthesis; previous researches analyzed the solasodine contents in several species of *Solanum* plants by using different methods failed in elucidating the main source of solasodine synthesis (Subroto and Doran, 1994).

Table 1 and 2 show that the highest percentage of solasodine contents in normal and transgenic plants of *S. nigrum* L. were found in stems and leaves, respectively. This is due to the difference in sample weights in each plant. In normal plants, stems are the heaviest plant part compared to other parts, thus the solasodine distribution in stems is higher. In transgenic plants, however, the highest solasodine percentage was found in leaves, this might be due to shorter of transgenic plants compared to the normal plants causing the solasodine was distributed to leaves faster. In addition, the transgenic plants relatively possess densed leaves

Table 1: Solasodine content in plant parts of *S. nigrum* normal plant

Sample	Retention time (min)	Area	Solasodine content (mg g^{-1})	Average solasodine content (mg g^{-1})	Total solasodine content (mg)	Average total solasodine content (mg)	Percentage of solasodine (%)
AN 1	15.1	171590	24.5	9.93	119.4	45.8	17.2
AN 2	-	ND	-		-		
AN 3	15.5	36739	5.3		18.0		
BN 1	14.5	29345	4.2	6.10	146.0	207.7	78.1
BN 2	14.7	97773	14.1		477.0		
BN 3	-	ND	-		-		
DN 1	14.6	36385	5.2	4.06	8.7	6.3	2.4
DN 2	14.962	33155	4.8		8.2		
DN 3	15.300	15344	2.2		2.0		
BuN 1	-	ND	-	0.61	-	6.0	2.3
BuN 2	-	ND	-		-		
BuN 3	14.504	12768	1.8		18.8		
					Total	265.7	

Notes:
Area of authentic solasodine (200 μg mL^{-1}) = 1211648
Total solasodine content = solasodine content×sample initial weight

$$\text{Percentage of solasodine} = \frac{\text{Average solasodine content}}{\text{Total solasodine content}} \times 100\%$$

AN 1 = Normal roots 1
AN 2 = Normal roots 2
AN 3 = Normal roots 3
BN 1 = Normal stems 1
BN 2 = Normal stems 2
BN 3 = Normal stems 3
ND = Not detected
DN 1 = Normal leaves 1
DN 2 = Normal leaves 2
DN 3 = Normal leaves 3
BuN 1 = Normal fruits 1
BuN 2 = Normal fruits 2
BuN 3 = Normal fruits 3

Table 2: Solasodine content in plant parts of *S. nigrum* transgenic plant

Sample	Retention time (min)	Area	Solasodine content (mg g^{-1})	Average solasodine content (mg g^{-1})	Total solasodine content (mg)	Average total solasodine content (mg)	Percentage of solasodine (%)
AT a	15.5	95280	13.6	7.75	10.7	19.7	23.3
AT b	15.1	36912	5.3		36.8		
AT c	15.1	30425	4.4		11.6		
BT a	14.4	32866	4.7	2.12	25.1	19.7	23.4
BT b	-	ND	-		-		
BT c	14.9	11643	1.7		34.0		
DT a	14.4	3510	0.5	3.98	3.4	43.6	51.6
DT b	14.5	79821	11.4		127.3		
DT c	-	ND	-		-		
BuT c	14.3	4981	0.7	0.71	1.3942	1.4	1.7
					Total	84.4	

Notes:
Area of authentic solasodine (200 μg mL^{-1}) = 1211648
Total solasodine content = solasodine content × sample initial weight

$$\text{Percentage of solasodine} = \frac{\text{Average solasodine content}}{\text{Total solasodine content}} \times 100\%$$

AT a = Transgenic roots a
AT b = Transgenic roots b
AT c = Transgenic roots c
BT a = Transgenic stems a
BT b = Transgenic stems b
BT c = Transgenic stems c
ND = Not detected
DT a = Transgenic leaves a
DT b = Transgenic leaves b
DT c = Transgenic leaves c
BuT c = Transgenic fruits c

compared to the normal plants (Subroto *et al*., 2001), thus solasodine was more distributed to the leaves. Therefore, it can be concluded that solasodine distribution in the transgenic plants was differ compared to the solasodine distribution in the normal plants.

Comparing between the normal and transgenic plants in term of their solasodine contents, every plant part of the normal plants contained higher solasodine compared to its counterpart in the transgenic plants (Table 1 and 2). This might be due to the difference in plant development and metabolism between the transgenic and the normal plants, such as longer flower development, fruit maturation, the lower numbers of flowers, fruits and seeds, etc. (Subroto, 1999; Subroto *et al*., 2001); this

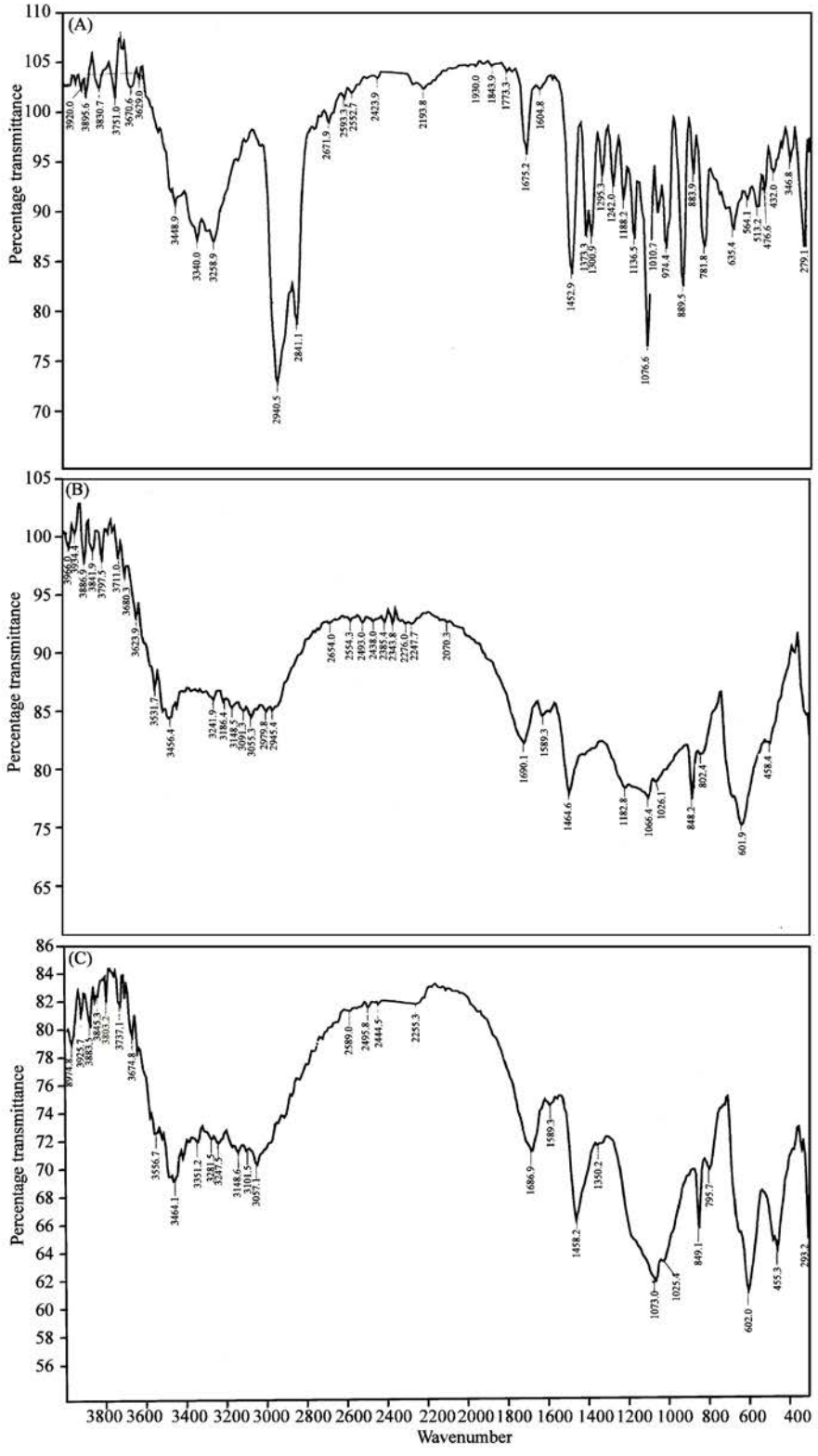

Fig. 1: IR spectra of solasodine. (A) Authentic solasodine; (B) Solasodine in normal plants and (C) : Solasodine in transgenic plants

Table 3: Summary of solasodine profiles in normal and transgenic plants analyzed by FTIR

	Wave number (ν cm^{-1})		
Functional group	Authentic solasodine	Normal plants	Transgenic plants
C-O	1070.6	1066.4	1073.0
C-N	1136.5	1182.8	1350.2
C-H	1452.9	1464.8	1458.2
	2940.5	2945.4	
C = C	1675.2	1690.1	1686.9
N-H	1640.8	1589.3	1589.3
	3258.9	3241.9	3247.5
O-H	3448.9	3456.4	3464.1

implies that at the same age (i.e., 16 weeks), the transgenic plants produced lower solasodine contents compared to its normal plants. Previous study showed that the presence of *rol* genes was detrimental to the alkaloid accumulation in the transgenic plants of *Hyoscyamus muticus* in contrast to hairy root cultures (Sevon *et al*., 1997). A similar condition is expected to cause the lower accumulation of solasodine in the transgenic plants in the present study. As shown in the previous study, the hairy roots used in the present study only contain T_L T-DNA (Subroto *et al*., 2001).

Table 1 and 2 also show that solasodine contents in each plant part, both in transgenic and normal plants, are varied considerably; even in some samples solasodine could not be detected by the HPLC method. One of the possible reasons is a phenomenon called somaclonal variation commonly found in plant tissue culture causing high diversity of plants obtained as well as their secondary metabolite synthesis (Subroto, 1999). Similar phenomena were also reported in transgenic plants of *Hyoscyamus muticus* derived from hairy roots (Sevon *et al*., 1997) and regenerated plants of *Atropa belladonna* transformed with *Agrobacterium rhizogenes* 15834 (Aoki *et al*., 1997). However, from the chromatograms obtained (data not shown), it can be concluded that the solasodine profile found in the transgenic plants is identical to the solasodine profile found in the normal plants. These findings are in contrast to the many published works on hairy roots which stated that hairy roots possess stable and high productivity of secondary metabolites (Guillon *et al*., 2006).

In addition to TLC and HPLC analyses, the solasodine profiles were also analyzed using infra red spectrophotometry. The results (Fig. 1 and Table 3) show that some functional groups presence in the authentic solasodine are also presence in the samples and the solasodine profile found in the transgenic plants is similar to the solasodine profile found in the normal plants.

CONCLUSIONS

The solasodine contents in plant parts of the transformed plants of *S. nigrum* were lower compared to that of the control plants. The solasodine contents in the control plants were 9.93 mg g^{-1} (roots), 6.10 mg g^{-1} (stems), 4.06 mg g^{-1} (leaves) and 0.61 mg g^{-1} (fruits), whereas the solasodine contents in the transformed plants were 7.75 mg g^{-1} (roots), 2.12 mg g^{-1} (stems), 3.98 mg g^{-1} (leaves) and 0.71 mg g^{-1} (fruits). Solasodine was differently distributed in transformed plants compared to the control plants. In control plants, the highest solasodine accumulation was occur in stems (78.1%), whereas in transformed plants the highest solasodine accumulation was occur in leaves (51.6%). It seems that the presence of *rol* (A, B, C and D) genes contained in the T_L T-DNA is detrimental to the solasodine accumulation in the transformed plants in contrast to transformed root cultures.

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