Korean J. Plant Res. 22(6) : 584～591 (2009)

Review

Establishment of *in vitro* Root Cultures and Analysis of Secondary Metabolites in Indian Ginseng - *Withania somnifera*

Neha G. Wasnik[1], Mahalakshmi. Muthusamy[1], Savitha Chellappan[1], Veena Vaidhyanathan[1], Ramakrishna Pulla[2], Kalaiselvi Senthil[1] and Deok Chun Yang[2]*

[1]Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam University for Women, Coimbatore-641043

[2]Korean Ginseng Center and Ginseng genetic Resource Bank, Kyung Hee University, Gyeonggi-do 449-701, South Korea

Abstract - Adventitious root culture was established in the Jawahar variety of *Withania somnifera* using MS basal medium supplemented with 0.5 (mg/l) IAA and 2.0 (mg/l) IBA. Root tips from germinated seedlings, MS0 maintained plants and adventitious roots were maintained in suspension medium (1/2 MS basal medium supplemented with 3% sucrose) for a period of 1 to 6 months. The weight gain in roots was noted and the withanolides were extracted from the dry roots using solvents petroleum ether, 50% ethanol and chloroform. The withanolides in the chloroform fractions of all root samples analyzed were compared using thin layer chromatographic analysis. Withanolide content in adventitious root sample was found to be superior compared to other roots at any given point of time during the 6month growth period.HPLC analysis of *in vitro* adventitious roots showed the presence of a new compound.

Key words - Adventitious roots, germinated roots, MS0, withanolides, TLC

Introduction

Medicinal plants play a key role in world heath care systems. Solanaceae is one of the largest families in the plant kingdom and comprises about 85 genera and more than 3000 species (Subbaraju *et al*., 2006). *Withania somnifera* is one of the most extensively used medicinal plants for adaptogenic purposes in Ayurvedic and Unani medicines belongs to this family (Roja *et al*., 1991). Most of its biological activities have been attributed to the presence of a group of compounds referred as withanolides present in roots and leaves of Withania and are used as drugs (Khanna *et al*., 2006). The root is regarded as a tonic, aphrodisiac and is used in consumption, emaciation, debility, dyspepsia and rheumatism. A decoction of the root is used for colds and chills (Davis and Kuttan, 2000). Since the roots contain a number of therapeutically applicable withanolides, mass cultivation of roots *in vitro* will be an effective technique for the large scale production of these secondary metabolites. The development of a fast growing root culture system would offer unique opportunities for producing root drugs in the laboratory without having to depend on field cultivation. Adventitious roots induced by *in vitro* methods showed high rate of proliferation and active secondary metabolism. Adventitious roots are natural; grow vigorously in phytohormone supplemented medium and have shown tremendous potentialities of accumulation of valuable secondary metabolites (Murthy *et al*., 2008). The withanolide content in each type of root of the plant has not yet been analyzed. In the present study, an attempt was made to establish suspension cultures of adventitious roots, roots from the MSO plantlets and germinated roots. This was followed by their growth for a period of 6 months, their harvest, extraction and analysis of the withanolide content in them.

Materials and Methods

Adventitious root induction

The healthy leaf explants from the plantlets maintained in MS basal medium were inoculated in adventitious root

*Corresponding author. E-mail : dcyang@khu.ac.kr

induction medium (MS basal medium supplemented with 0.5 mg/l IAA and 2.0 mg/l IBA) under 16hr phtotperiod.

Roots employed and their establishment in suspension

For *in vitro* mass cultivation of roots, three types of root explants were chosen, 1 month old germinated seedlings 1 month old well grown MS0 maintained plants and 1 month old adventitious roots. The root tips (about 25-30) were inoculated in hormone free ½ MS suspension medium with 3%sucrose. The cultures were maintained at 22℃ at 80-90 rpm. The roots were regularly supplied with fresh medium once every 28 days. The grown roots were harvested every month over the 6 month period. Seedlings germinated in green house were maintained under recommended growth conditions and roots were harvested after 3 months for analysis.

Extraction of withanolides

Modified method of Khajuria *et al*., (2004) was followed for the extraction of withanolides. One gram of dried root was defatted with 20 ml of petroleum ether (60°-80℃). To this residue, 20 ml of 50% ethanol was added and extraction was carried out. To the ethanol fraction 15 ml of chloroform was added and subjected to further extraction. The chloroform and the ethanol layers were collected separately. All the solvent fractions were evaporated, dried and reconstituted with HPLC grade methanol.

Thin Layer Chromatography and analysis of withanolides

The stored samples were analyzed by TLC using Silica gel 60 F254 plates of size 7 cm X 5 cm. 20 µl of samples were spotted on the TLC plate, air-dried and placed in a chromatographic chamber saturated with solvent. After spotting each sample, the plate was placed in a saturated chamber containing the solvent system Chloroform:Methanol in the ratio 9:1 for adventitious roots. The plate was dried, and dipped in 10% sulphuric acid. This was followed by incubating the plates in a hot air oven until spots appeared. The Rf values were also calculated to analyze the various components in root samples and compared with that of withanolidde-A standard.

HPLC analysis of *in vitro* germinated and *ex vitro* roots

A gradient HPLC (Shimadzu HPLC Class *VP* series) with two LC- 6 AD pumps (Shimadzu), variable wavelength programmable photo diode array detector SPD M10A *VP* (Shimadzu), CTO-10AS *VP* column oven (Shimadzu), CBM-10A *VP* system controller (Shimadzu), and reverse phase C18 phenomenex column (250 mm × 4.6 mm), were used. The HPLC system was equipped with Class *VP* series version 6.1 software (Shimadzu).

The mobile phase used for the analysis consisted of acetonitrile and water, which were applied in the following gradient elution: from 50:50 in 0 min, 0:100 in 20 mins, 50:50 in 30 mins and finally 100:0 in 32 mins. The flow rate for the binary HPLC run was kept 1.0 ml/min and the run was continued for 32 mins for complete resolution and detection of the withanolides at 225 nm using a PDA detector, which yielded a column backup pressure of 135 to 145 kgf/cm^2. The results were compared with the HPLC pattern of the three months old *ex vitro* roots and 6 month old *in vitro* roots.

Results and Discussion

Roots play vital roles in whole-plant growth and crop productivity. They are also a source of secondary metabolites, especially in plants producing pharmaceutical compounds in their roots. The leaves of *Withania somnifera* were inoculated in MS basal medium supplemented with 0.5 mg/l IAA and 2.0 mg/l IBA. The roots began to initiate between 9-12 days. The response of the explants to the formation of adventitious roots was found to be 98%. The length of the root was between 0.4-0.8cms at the end of 15-day observation period. The length of the roots was similar to that observed by Rani *et al*., (2003). The hormones IBA, IAA and NAA were employed and maximum of 65% of root induction was observed in their cultures. However, the percentage of cultures giving rise to roots was much higher in the present study. The stages of adventitious root formation are represented in Fig. 1.

As compared to the complexity of the *Agrobacterium rhizogenes* mediated transformation for the induction of hairy roots; adventitious root induction is more effective. For the establishment of roots in suspension, the root tips of each type of root were individually taken. About 25-30 root tips were

uniformly inoculated in a culture bottle containing 30 ml of liquid ½ MS basal medium containing 3% sucrose. Since root tips are auxin concentrated regions where active cell multiplication takes place, they were chosen for the establishment of suspension cultures. Regular replacement of medium at 28 days interval was done in order to supply adequate nutrients for growth. The increase in weight of the root cultures is presented in Table 1.

At the end of 30 days, it was observed that all the types of roots increased in length. The adventitious roots showed maximum growth among the different types. It also showed more branching when compared to germinated and MS0 obtained roots. The germinated roots generally only elongated, with very less branching. The doubling time of each type of root was between the 30 and 60-day period. The roots were healthy, white in colour. Towards the end of the 60-day

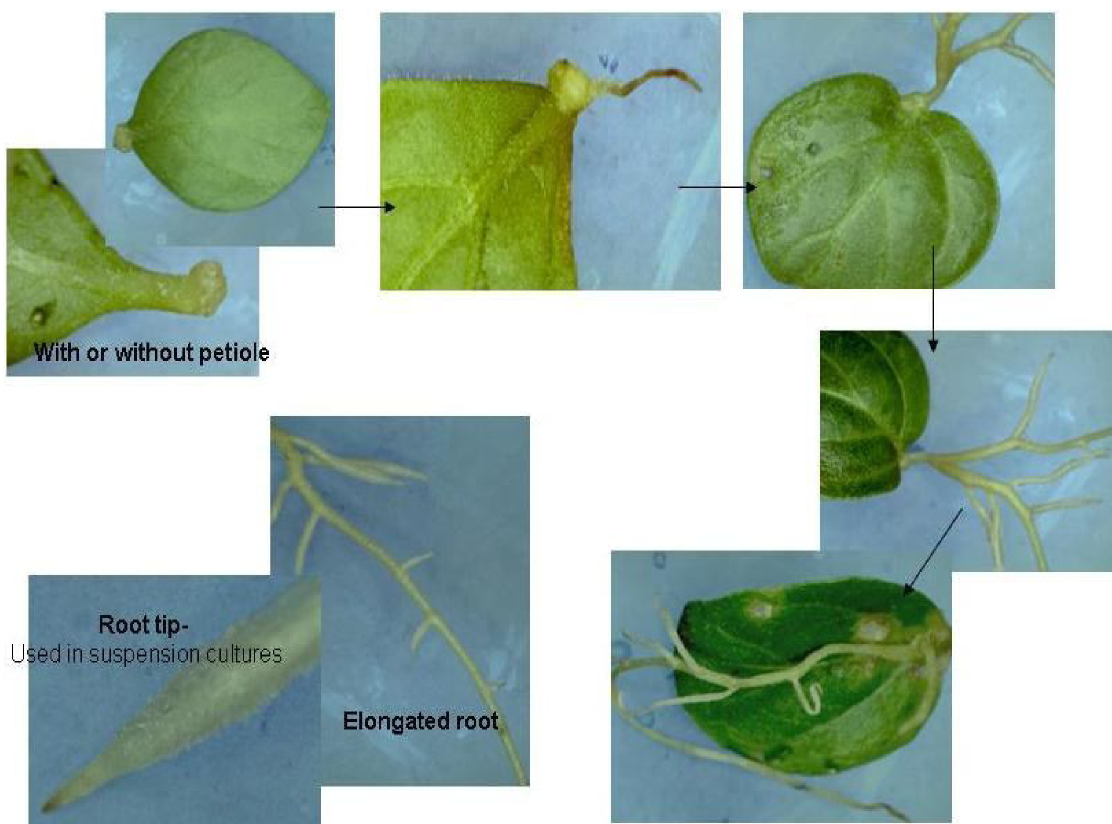

Fig. 1. Adventitious root induction in *W. somnifera*.

Table 1. Mean weight of the three types of roots in suspension over 6-month period

S. NO	PERIOD OF GROWTH	TYPE OF ROOT IN SUSPENSION (WEIGHT IN GRAMS)		
		GERMINATION	ADVENTITIOUS	MSO
1.	6 months	3.16	6.95	5.62
2.	5 months	3.37	6.79	5.61
3.	4 months	2.83	5.78	4.54
4.	3 months	1.90	4.67	3.01
5.	2 months	0.83	3.74	1.73
6.	1 months	0.45	**1.83**	0.96

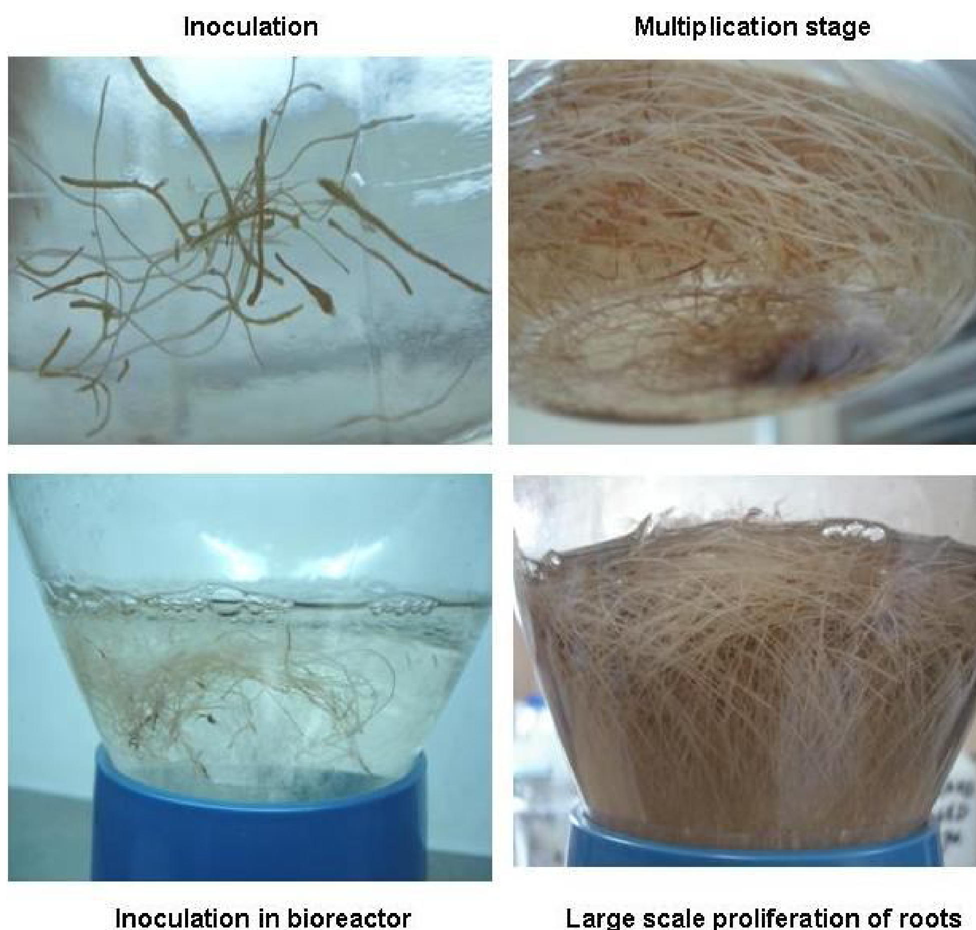

Fig. 2. Different stages of roots in suspension.

period, the older parts of the roots i.e., the ones that had been initiated first start turning slightly pale yellow in colour. The different stages of root growth in suspension using bioreacor are represented in Fig. 2. By the end of 90 days the older parts of the root start to turn pale golden yellow in colour and as time progresses the colour intensity was found to increase. After a point of time the roots stop growing further. Also at this point of time the roots are brown in color and also brittle in nature. When compared to germinated and MS0 derived roots, the adventitious roots remain healthy for a longer period of time.

Extraction of withanolides

Extraction of withanoldes was done using different solvents; petroleum ether, 50%ethanol and chloroform. Chaurasiya *et al.* (2007) used fresh roots ground in liquid nitrogen and extracted overnight in 20 ml of methanol: water (25:75) at room temperature. The filtrates were pooled and extracted with n-hexane (3 x 60 ml). The n-hexane fraction was then discarded and the methanol-water fraction was further extracted with chloroform (3 x 60 ml). The chloroform fractions were pooled and concentrated to a dry powder. This was used for further analysis. For dried, powered roots of withania ethanol:water (1:1) was used and percolated 4-times at room temperature. The four extracts were combined, centrifuged, and finally concentrated to 1/8 of the original volume under reduced pressure at 50 *l* 58C. The concentrated extract was thoroughly extracted with chloroform. The chloroform extract was distilled under reduced pressure to yield a residue (Khajuria *et al*., 2004). Though many extraction procedures have been followed, we have chosen the procedure close to the above mentioned references for use in the present study for the efficient extraction of withanolides from *in vitro* roots of Jawahar variety of *W. somnifera*.

Thin layer chromatography of withanolides

The petroleum ether fraction of samples collected over 6 month period of adventitious roots, germinated roots and MSO derived roots were spotted. The Rf values obtained at individual lanes are shown in the respective plate. The spots with Rf value 0.12, 0.19, 0.53, 0.58, 0.80 and 0.88 were observed among the samples. The spots with Rf value 0.12 and 0.19 were present in adventitious root sample of 1, 2 and 3 month old roots and 1month old roots of MSO roots. The spots at Rf value 0.53 and 0.58 were noted in adventitious root sample of 1, 2 and 3 month old roots. The intensity of 0.53 and 0.58 was highest at 3 month and 1 month respectively. These spots are not well resolved in germinated roots. 1 and 2 month of MSO roots showed the spot with Rf value 0.58.The ethanol fraction of the roots did not yield any proper resolution of spots. After the addition of chloroform to this fraction, no lanes are visible. The dark spots at the origin indicate the presence of sugars. As a result of 50% ethanol fractionation, the sugars present in the root samples are successfully removed.

In the final step of the analysis procedure, the chloroform fractions of the various root samples are subjected to TLC analysis. The chromatogram is characterized by good resolution of spots. The spots are distinct and clear and maximum number of spots is obtained in the chloroform fraction when compared to the other solvent fractions. The Rf values obtained are compared and presented in Table 2 and the thin layer chromatograms in Fig. 3. The Thin Layer chromatograms showed Rf values namely 0.11, 0.19, 0.26, 0.30, 0.33, 0.42, 0.51, 0.55, 0.58, 0.64, 0.70, 0.8 and 0.88. There was variation in the number of spots obtained both according to the age of the root and also according to the type of the root. In the 1 month old roots, adventitious roots had a maximum number of 10 spots followed by MS0 derived roots showing 5 spots and germinated roots showing only 3 spots. This indicates the early synthesis of withanolides in the adventitious roots when compared to germinated or MS0 roots. The spot with Rf value 0.11 was detected in all root samples over the 6 month period except in 1 month old germinated roots. The spot with Rf value 0.19 and 0.26 was found in all root samples at all times. The spot at 0.3 was detected only from the 2nd month. However, this spot was also detected in the 1 month old root sample of adventitious roots. The spot with Rf value 0.33 was found only in 1 and 2 month old root samples. The spot with Rf value of 0.42 was detected in all root samples from the 3rd month. In the process of development,

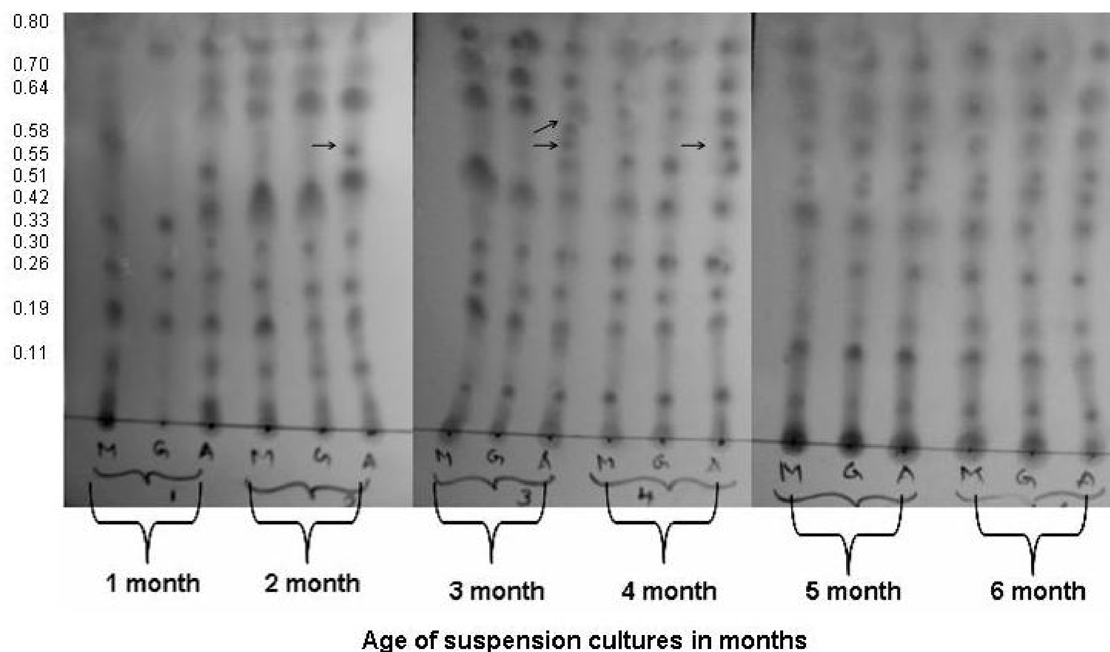

Fig. 3. TLC chromatogram showing the comparison of withanolides in different types of roots over 6 month period.
M - roots from MS0 maintained *in vitro* plantlets, G - roots from germinated seedlings, A - roots from adventitious roots.

Table 2. Rf values of chloroform fractions of different types of root samples in Jawahar variety of *Withania somnifera*

Month	Root type	Rf value												No. of spots
		0.11	0.19	0.26	0.3	0.33	0.42	0.51	0.55	0.58	0.64	0.7	0.8	
1	M	*	*	*		*					*			5
	G		*	*		*								3
	A	*	*	*	*	*		*			*	*	*	9
2	M	*	*	*	*	*		*			*	*	*	9
	G	*	*	*	*	*		*			*	*	*	9
	A	*	*	*	*	*		*	*		*	*	*	10
3	M	*	*	*	*		*	*			*	*	*	9
	G	*	*	*	*		*	*			*	*	*	9
	A	*	*	*	*		*	*	*	*	*	*	*	11
4	M	*	*	*	*		*	*			*	*	*	9
	G	*	*	*	*		*	*			*	*	*	9
	A	*	*	*	*		*	*	*		*	*	*	10
5	M	*	*	*	*		*	*	*		*	*	*	10
	G	*	*	*	*		*	*	*		*	*	*	10
	A	*	*	*	*		*	*	*		*	*	*	10
6	M	*	*	*	*		*	*	*		*	*	*	10
	G	*	*	*	*		*	*	*		*	*	*	10
	A	*	*	*	*		*	*	*		*	*	*	10

* Indicates presence of spot M=MSO roots G=Germinated roots A=Adventitious roots

this may probably indicate the conversion of lower withanolides into higher ones as indicated here by the disappearance of spot and 0.33 and appearance of a spot at 0.42. The spot at 0.51 was found in all root samples from the age of 2 months. However, this spot was present only in the one month old adventitious root but was absent in MS0 and germinated roots. The spot at 0.55 was present 2, 3 and 4 month old adventitious root sample. Later this spot was visible in the 5 and 6 month old samples of all types of roots.

The presence of spot with Rf value of 0.64 was found in all root samples except for one month old germinated root sample. The higher spot of Rf values 0.7, 0.8 and 0.88 are deduced in all root samples from 2 to 6 months. These three particular spots are found only in the adventitious root samples among the one month old roots. Generally it can be noted that the withanolide spots start appearing at an early age in the adventitious root sample in comparison with germinated and MSO roots. This is proven by 10 spots being present in 1 month old adventitious root sample when compared to only 5 and 3 in MSO and germinated root sample respectively. Also the number of withanolides spots visible at any given point of time is highest in the adventitious root samples. Earlier also withanolides from *in vitro* cultures have been analyzed. Sharada *et al*., (2007) found that *in vitro* cultures established from leaf accumulated the highest level of withanolides and those from shoot tips of well-grown plants produced the lowest level which suggested that tissue cultures having different morphology showed the inherent biosynthetic capability of the donor plant under in vitro conditions.

HPLC analysis of withanolides

When the chromatograms of the various samples were compared (Fig. 4), it was found that a similar pattern of elution of peaks occurred between 4.3-4.7mins for standard Withanolide-A, *in vitro* 3 month old roots and 3 month old *in vivo* roots. The chloroform extract of *in vitro* 3 month old

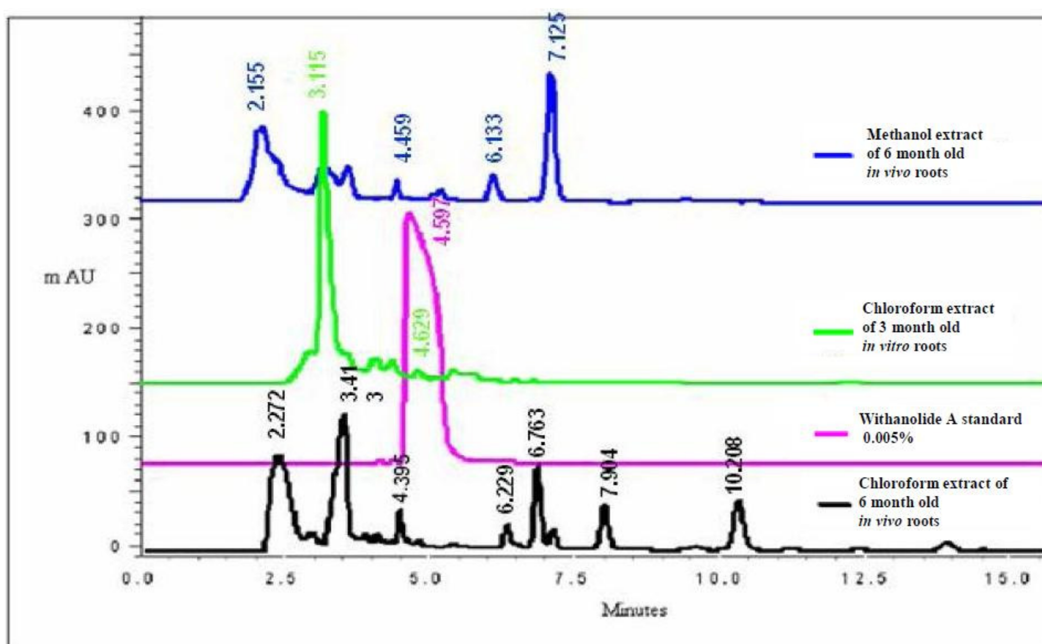

Fig. 4. Chromatogram overlays of different root samples.

roots showed a new compound eluted at 3.115 min. When compared to TLC pattern of chloroform extracts of adventitious root, the solitary spots found in one and two month old samples may be compared to this single major peak. These observation leads to the speculation that new intermediate compounds may be formed during the development of major withanolides. The area counts of the eluted peaks were also determined which helped to determine the concentration of the compounds.

Dalavayi *et al*., (2006) investigated the marker compound Withaferin-A in different parts of *Withania somnifera* (roots and leaves) as well as the area count for different concentration of the standard and reported that the variation in retention time of peak of Withaferin-A in chromatograms of *W. somnifera* may be due to the presence of other chemical constituents. The concentration calculated using the standard for different samples showed variation in withanolide content. Mature roots were found to give 0.0009% and immature *ex vitro* roots gave 0.00011% of Withanolide-A for 20 µl of the sample. The amount of various constituents was found to increase with the age of the roots and with the supplementation of extra nutrients.

Further analysis involving large scale purification of compound and structural studies of the single major peak identified in 3 month old *in vitro* roots are essential which will provide information on the biosynthetic pathway of withanolides.

Acknowledgements

INSA-KOSEF fellowship provided to Kalaiselvi Senthil is gratefully acknowledged. Also, this work was supported by Plant Diversity Research center of 21st Century Frontier Research Program (Code #PF06222-00).

Literature Cited

Wadegaonkar, P.A., Bhagwat, K.A. and Rai, M.K. 2006. Direct rhizogenesis and establishment of fast growing normal root organ culture of *Withania somnifera* Dunal. Plant Cell, Tissue and Organ Culture. 84: 223-225.

Uurthy, H.N., Dijkstra, C., Anthony, P., White, D.A., Davey, M.R., Power, J.B., Hahn, E.J. and Paek, K.Y. 2008. Establishment of *Withania somnifera* hairy root cultures for the production of Withanolide A. Journal of Intergrative Plant Biology 50(8): 975-981.

Davis, L. and Kuttan, G. 2000. Immunomodulatory activity of *Withania somnifera*. Journal of Ethnopharmacology 71: 193-200.

Khanna, P.K., Kumar, A., Ahuja, A. and Kaul, M.K. 2006. Biochemical composition of roots of *Withania somnifera* (L.) Dunal. Asian Journal of Plant Sciences 5: 1061-1063.

Khajuria, R.K., Suri, K.A., Gupta, R.K., Satti, N.K., Amina, M., Suri, O.P. and Qazi, G.N. 2004. Withanolides in plant extracts of *Withania somnifera* by HPLC-UV(DAD)-positive ion electrospray ionisation- mass spectrometry. J. Sep. Sci. 27: 541-546.

Rani, G., Virk, G.S. and Nagpal, A. 2003. Callus induction and plantlet regeneration in *Withania somnifera*. In Vitro Cell. Dev. Biol. 39: 468-474.

Chaurasiya, N.D., Uniyal, G.C., Lal, P., Misra, L., Sangwan, N.S., Tuli, R. and Sangwan, R.S. 2008. Analysis of withanolides in root and leaf of *Withania somnifera* by HPLC with photodiode array and evaporative light scattering detection. Phytochemical Analysis 19(2): 148.

Sharada, M., Ahuja, A., Suri, K.A., Vij, S.P., Khajuria, R.K., Verma, V. and Kumar, A. 2007. Withanolide production by *In vitro* cultures of *Withania somnifera* and its association with differentiation. Biologia Plantarum 51: 161-164.

Dalavayi, S., Kulkarni, S.M., Itikala, R.L. and Itikala, S. 2006. Determination of Withaferin-A in two *Withania Speciesby* RP-HPLC Method. Indian Journal of Pharmaceutical Sciences 68: 253-256.