WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 6.647
Volume 6, Issue 8, 842-853 Research Article ISSN 2278 – 4357

A STUDY ON THE INFLUENCE OF PLANT GROWTH REGULATOR ON SHOOT MULTIPLICATION AND EVALUATION OF MAJOR WITHANOLIDES IN *WITHANIA* somnifera

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Article Received on
08 June 2017,

Revised on 27 June 2017,
Accepted on 18 July 2017,

DOI: 10.20959/wjpps20178-8944

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ABSTRACT

W. somnifera is one of the top range medicinal herb with a rich repository of pharmaceutically active steroidal lactones known as withanolides. In the present study, adventitious shoot multiplication protocol of *W. somnifera* was optimized in media supplemented with different concentration of cytokinin (BAP and Kin). The maximum number of shoots (10.2±0.86) was observed in MS basal medium supplemented with BAP (4.44µM), 3% sucrose and 0.8% agar and the same was found to be most suitable medium for *in vitro* adventitious shoot multiplication (28 ± 0.89) in suspension. An increase in shoot mass of 38.6g/L and presence of bioactive compound withanolide A (129.18±0.33) and withaferin A (968.6±0.45) was observed in *in vitro* shoot that was analyzed using HPTLC. *In vitro* adventitious shoots in bioreactors could be used as a quick and efficient process of generating multiples that would offer unique opportunities for producing shoot based pharmaceutical products without having to depend on field cultivation.

KEYWORDS: *Withania somnifera*, adventitious shoots, BAP, Kin, withanolides, HPTLC.

INTRODUCTION

Withania somnifera belonging to the family Solanaceae is a plant of immense medicinal value largely due the presence of array of bioactive withanolides. *W. somnifera* which also

known as ashwagandha is an extensively used medicinal herb in the Indian traditional system of medicine. It is an annual self fertilizing herb with restricted geographic distribution growing exclusively as small-cultivated populations only in drier regions of India.[1] This plant is included in GRAS (‘generally regarded as safe’) category and its dried leaves and roots are included in more than100 formulations in Ayurveda, Siddha and Unani systems of medicine[2] and help in recovery from neurodegenerative and neuropsychiatric central nervous system disorders, endocrine and cardiovascular dis-orders, cognition facilitating, antiageing properties principally attributed to the steroidal lactone withanolides.[3,4]

The major components responsible for these biological activities are the Withanolides which are credited with widely acclaimed remedying properties. A group of naturally occurring C28 steroidal lactones are built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring. The basic structure is designated as the withanolide skeleton. Withanolides are known as plant hormones, which can be used instead of physiological human hormones. Withanolides are amphiphilic compounds which are able to regulate activities and the physiological body hormones processes. According to a theory, when these plant hormones enter the human body, they occupy the active receptor of the cell wall and don’t allow the animal hormones to get binding to this site and express their true activities. (Alternative Medicine Review, Monograph, 2004). At present, more than 12 alkaloids, 40 withanolides, and several sitoindosides (a withanolide containing a glucose molecule at carbon 27) have been isolated and reported from aerial parts, roots and berries of *Withania* species.[5,6] Major Withanolides, like withaferin A and withanolide A of the plant have been demonstrated to possess significant therapeutic actions.[7] Among them one of the most important withanolides isolated from Withania extracts is the anticancer compound withaferin A.[8]

The availability of *W. somnifera* is becoming scarce due to an increase in market demand for its slow growth rate in its natural habitat and poor seed viability. The annual requirement of Withania drugs in India has been estimated about 9,127-tonnes while the annual production is 5,905-tonnes. So necessitating to increase its cultivation and higher production.[9] In this case, Plant tissue culture techniques provided an excellent opportunities for *Withania* based medicinal production to meet a growing demand of pharmaceutical industries. Over recent decades, several attempts have been made to improve withanolide production through tissue culture.[10] Production of active principles through *in vitro* shoot suspension has gained

considerable attention in recent years, and such methods are increasingly attractive alternatives to whole plant cultivation for production of high value phytochemicals.[11,12] *In vitro* adventitious shoots in bioreactors could be used as a quick and efficient process of generating multiples that would offer unique opportunities for producing shoot based pharmaceutical products without having to depend on field cultivation.

The *in vitro* shoot suspension culture used in many ways to improved secondary metabolite production, such as the optimization of culture conditions, the selection of high producing strains of lines, precursor feeding, elicitation, metabolic engineering, transferring root cultures, micropropagation and bioreactor cultures among others.[13] In an earlier publication, it was reported the development of a shoot suspension culture system for *W. somnifera* that resulted in improved biomass and withanolide production.[14] Further enhancement can also be achieved by manipulation of the medium composition such as hormonal combination on shoot suspension culture of W. *somnifera*. A readily available sucrose and nitrogen seems to be important to maintain cultured cells. Both growth and morphogenesis in tissue culture are markedly influence by the availability of sucrose and nitrogen source and the form in which it is presented.[15]

Therefore, the present study was achieved to investigate the influence of hormonal combination on large scale production of biomass and withanolide accumulation in *W. somnifera.*

MATERIALS AND METHODS

Materials

Plant materials: Seeds of a released variety of *Withania somnifera*; Jawahar Asgandh-20 were purchased from Tamil Nadu Agricultural University (TNAU), Coimbatore. Surface sterilized seeds were then germinated *in vitro* in Murashige-Skoog (MS) media supplemented with 2% sucrose and the seedlings produced were maintained on MS basal medium.

Explants

Shoots originated from *in vitro* germinated seedlings of *W. somnifera*, were subcultured in MS basal media and were maintained for two months sub-culturing periodically (15-20 days interval). Fresh and healthy nodal sections were inoculated on to MS solid medium containing 2.22µM BAP used for shoot multiplication. This hormonal combination was selected on the basis of our earlier report. The 15 days old *in vitro* solid medium with 4.44

µM BAP containing growing shoot cluster were transferred into suspension media for standardization of shoot multiplication, biomass accumulation and withanolides production.

Methods

Standardization of *in vitro* culture system for *Withania somnifera*

Media Preparation and Sterilization

The MS basal medium[16] was used for micro propagation, root induction and suspension culture studies. The composition of the medium is given in **Table 1**. (Milli–Q) water was used for the preparation of culture media. The growth regulators at required concentration were added. The pH of the media was adjusted to 5.6- 5.8 using 0.1 N NaOH or 0.1 N HCl. Gelling agent (agar-agar Type-I) at a concentration of 0.8% was added and the medium was steamed to melt the gelling agent. It was then dispensed into culture vessels (30 ml per bottle) or liquid medium without gelling agent into conical flasks (100 ml per 250 ml flask). The medium was autoclaved at 15 lbs pressure at 121° C for 20 min.

Optimization of plant growth regulators on shoot multiplication, biomass and withanolides production in *W. somnifera*

The 15 days old *in vitro* solid medium with 4.44 µM BAP containing plantlet used as an explants for present study. The culture were established with inoculums mass of 2g FW shoot cluster consisting of average of 1 cm shoot length and 6-8 number of shoots with 1-2 nodes were transferred in 20 ml MS basal media supplemented with different concentrations of hormones 6-Benzylaminopurine (BAP) and Kinetin (Kin) *w*ere used. The media used with varying hormone combinations are given in the **Table 1** The explants were then cultured at 25ºC and observed regularly for contamination or for any other morphological changes. Each experiment had 4 replicates. A photoperiod of 16/8 hrs was maintained for all experiment. Finally the accumulation of multiple shoots, biomass and withanolides production was observed after 30 days of culture.

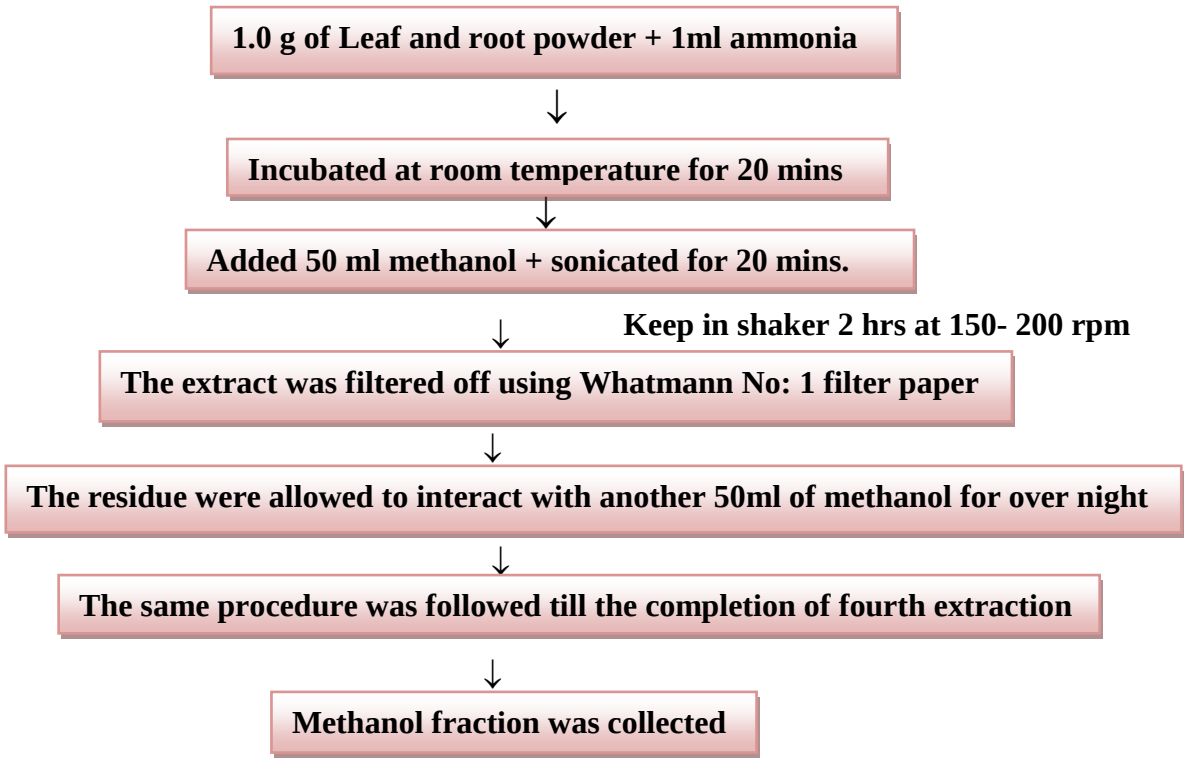
Table. 1 Media composition used for multiple shoot induction

S. No.	Treatments	Media composition (µg/L)	
		BAP	KIN
1.	T0	0	0
2.	T1	2.22	0
3.	T2	4.44	0
4.	T3	8.88	0
5.	T4	0	2.32
6.	T5	0	4.64
7.	T6	0	9.28
8.	T7	2.22	2.32
9.	T8	2.22	4.64
10.	T9	2.22	9.28
11.	T10	4.44	2.32
12.	T11	4.44	4.64
13.	T12	4.44	9.28
14.	T13	8.88	2.32
15	T14	8.88	4.64
16.	T15	8.88	9.28

Quantitative Analysis of Secondary Metabolites

Extraction of Secondary Metabolites

One gram of dried *in vitro* shoot powder of *Withania somnifera* was taken and the evaluation of secondary metabolites was carried out as indicated in the following flow chart.[17]



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Dissolved in HPLC grade methanol

↓

Quantification for HPTLC

Preparation of standards: Standard solutions of withanolide A and withaferin A (1.0mg/ml) were prepared using HPLC grade methanol and stored in a refrigerator at 4°C. From the stock solutions, working solutions were prepared by dilution with HPLC grade Methanol.

Quantification of Major Withanolides: HPTLC was performed on precoated silica gel aluminum plate (20 cm X 20 cm) 60F254 (E.MERCK, Germany). The methanolic extract of *W. coagulans* were loaded to the plates as 6 mm bands, under a stream of nitrogen using the CAMAG (Switzerland) Linomat V semiautomatic sample applicator fitted with a 100 µl of Hamilton HPTLC syringe. The HPTLC plates were developed up to 80mm using the mobile phase Toluene: Ethyl Acetate: Formic acid in the ratio of 5:5:1 respectively in a Camag Twin trough glass tank. It was pre saturated with the mobile phase solvents for 30 minutes at room temperature (25± 2 ºC). The developed plate was air dried and the image was captured at 245 nm and 366 nm. Densitometric scanning was performed at 235 nm for withanolide A and 530 nm for withaferin A using Camag TLC scanner III controlled by CAMAG CATS 4 integration software. The Rf values of the resolved spots were noted. The peak areas were evaluated using linear regression.[18]

The amount of withaferin A and withanolide A in the samples were quantified using peak area. The plates were derivatized using anisaldehyde sulphuric acid (85mL methanol: 10mL glacial acetic acid: 5mL sulphuric acid: 0.5mL anisaldehyde) reagent and placed in hot-air oven for 2 min at 110°C for detection of spots.

RESULTS AND DISCUSSION

Effect of plant growth regulators on shoot multiplication, biomass and withanolide accumulation: Plant growth regulators are chemical substance that regulate plant growth and development and also produced signal molecules within the plant, and occur extremely low concentrations. In previous study, many authors has been used solid medium for shoot multiplication in *W. somnifera.*[19,20,21,14] However, in shoot culture, the shoot proliferation has declined due to the use of solid medium. Hence, in this scenario a very recently find out the liquid medium has been selected for mass production of shoots. Different concentration and

combination of plant growth substance were used to optimize the best media combination for shoot proliferation in suspension culture of *W. somnifera*. A maximum number of multiple shoots were observed in medium with 4.44µM BAP (38 ±1.09) and 2 g l^{-1} fresh weight of shoot inoculums mass were used to find out maximum biomass growth (38 g/L) in *W. somnifera (*Fig 1 and 2).

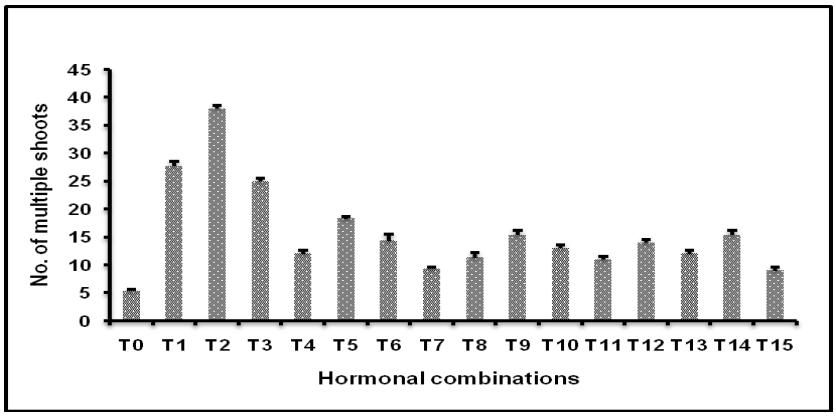


Fig1. Influence of plant growth regulators on shoot proliferation in suspension culture of *W. somnifera*

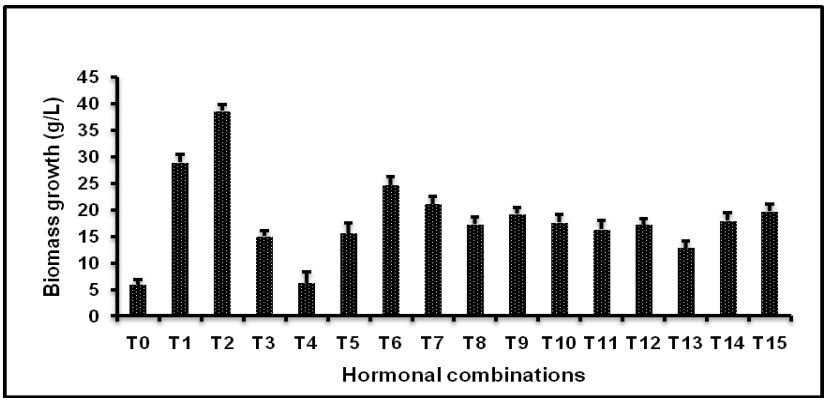


Fig2. Influence of plant growth regulators on biomass growth in suspension culture of *W. somnifera*

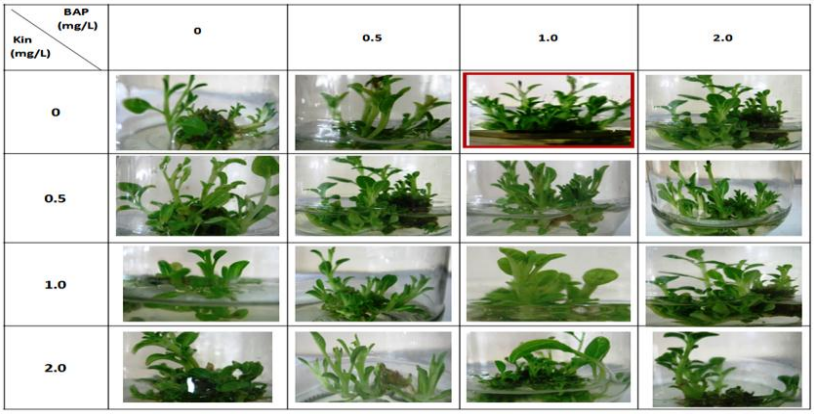


Plate 1. Optimization of Plant growth regulators on shoot multiplication in suspension

In present study, exogenous supplement of plant growth substance were increase the multiple shoots and secondary metabolites production in medium with 4.44µM BAP. Our results entirely different from Sivanandhan *et al.* (2013), he reported that enormous shoot proliferation and withanolide production in medium with 0.6 mg BAP and 20µM polyamines in *W. somnifera* .The culturing of the shoot cluster with higher concentration of Cytokinins has led to altered morphology of multiple shoots. The same response were observed[22] in *Hypericum hirsutum* and *H. maculatum and*[14] in *W. somnifera* liquid culture. Above the result it was concluded that low concentration of Cytokinins led to more vigorous shoots and there is no hyperhydricity or necrosis.

The previous study[23] observed (7.0±0.89) shoots and (5.3±0.26) shoots /explants on MS solid medium amended with TDZ and BAP respectively and[24] reported that maximum number of multiple shoots observed on solid medium with combination of 6.0µM BAP and 0.2 NAA (35.70± 0.97) in *Oldenlandia biflora L.*[25] observed that large number of multiple shoots on solid medium with 0.5 mg/L and 0.2 mg/L NAA (8.2 ± 0.66) in *Luffla acutangula (L) Roxb*. But a more recent research[14] found out enomours number of shoot proliferation on MS liquid medium amended with low concentration of BAP. He reported that maximum number of multiple shoot on MS required medium with 0.6 µM BAP (86.27±2.0) in *W. somnifera.*

The quantity of withanolide A (129.18±3.1) and Withaferin A (968.6±6.1) contents of proliferated shoots in suspension medium varied with in treatments (Fig 3 and 4). The range of withanolide A content from control to treated sample were 61.44±1.21 to 129.18±3.1 and Withaferin A 518.22±3.21 to 968.6±6.1 in proliferated shoots on the medium with 4.44µM BAP after 20 days of culture. The amount of these compounds increased to 2.11 fold (withanolide A) and 1.86 fold (withaferin A) higher when compared to control. The lowest content of withanolide accumulation was found in medium with Kin used as alone. It has been clearly indicate that concentration of plant growth regulators is highly influence for the production of secondary metabolites in shoot culture of *W.somnifera.*[26,9]

The higher amount of withanolide content was achieved on medium with BA and coconut water in shoot suspension culture of *W. somnifera (*Ray and Jha,2001) and also the maximum withanolide accumulation was observed on medium supplemented with BAP and spermidine (Sivanandhan, et al., 2013) . But our present investigation, without any addition on medium

amended with plant growth regulator alone produced higher amount of withanolide content in shoot suspension of *W. somnifera*.

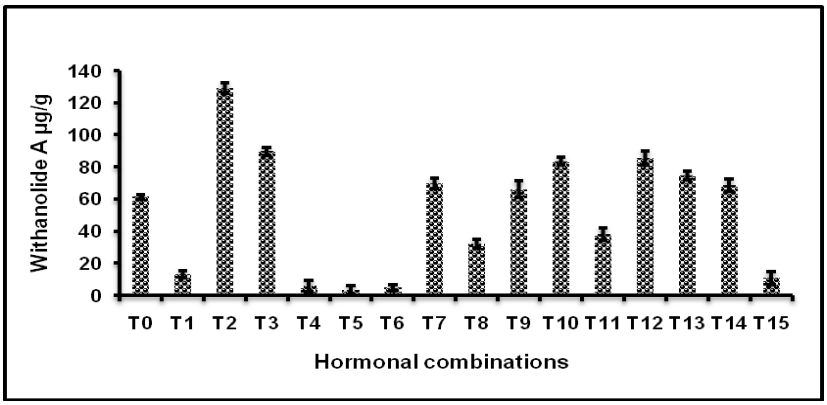


Fig3. Influence of plant growth regulators on withanolide A production in suspension culture of *W. somnifera*

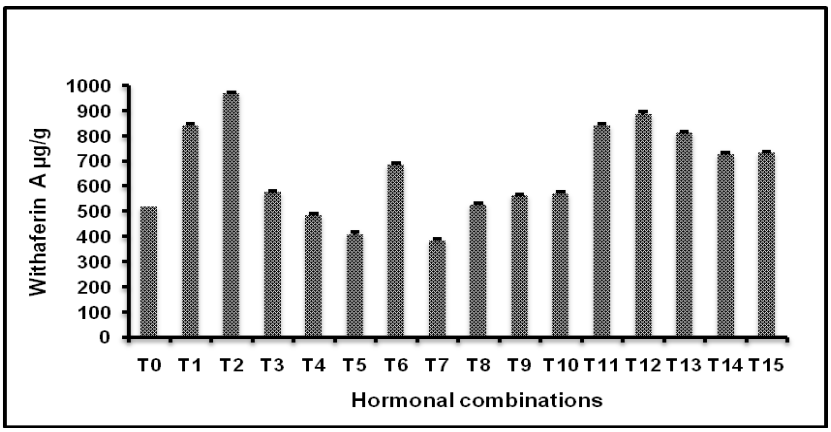
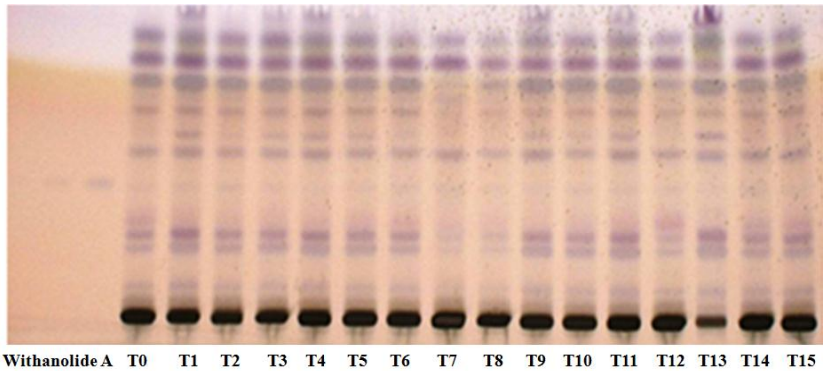


Fig4. Influence of plant growth regulators on withaferin A production in suspension culture of *W. somnifera*



Solvent system : Toluene : Ethyl acetate: Formic acid (5:5:1)
Under white light after dervatizing with anisaldehyde sulphuric acid

Plate 2. HPTLC banding pattern of withanolide A on hormonal treated *in vitro* shoot sample

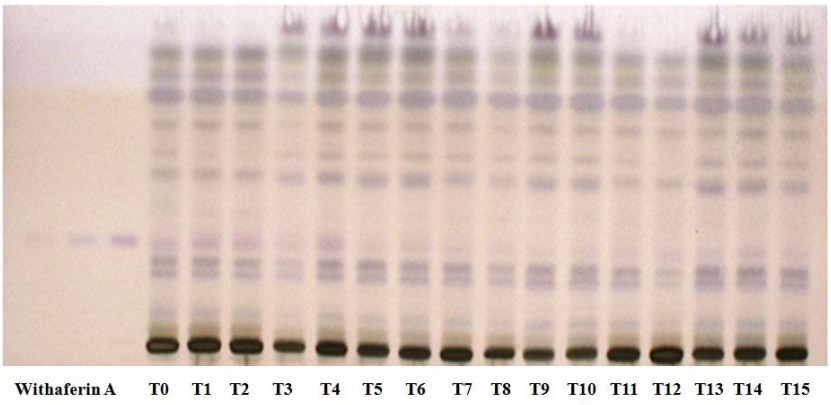


Plate 3. HPTLC banding pattern of withaferin A on hormonal treated *in vitro* shoot sample

CONCLUSION

Culturing of *in vitro* adventitious shoots was established from *in vitro* grown shoots of *W. somnifera*. Nearly 2.11 fold increase in Withanolide A accumulation and 1.86 fold increase in withaferin A accumulation was observed than the control un treated shoot samples when the medium was supplemented with 4.44µM BAP. This *in vitro* shoot multiplication regeneration system could provide unique opportunities for large scale production of shoot tissues as an alternate to field grown tissues for a constant supply of raw material with standardized withanolide content.

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